ACTA AGRICULTURAE SLOVENICA

116 • 1
2020

Biotehniška fakulteta Univerze v Ljubljani
Biotechnical Faculty University of Ljubljana

Acta agriculturae Slovenica • ISSN 1581-9175 • 116–1 • Ljubljana, september 2020

Acta agriculturae Slovenica
Volume / Letnik **116** · Number / Številka **1** · **2020**

VSEBINA / CONTENTS

doi:10.14720/aas.2020.116.1.1648 Review article / pregledni znanstveni članek

Cold stress at seedlings stage of buckwheat optimizes development of both roots and aboveground biomass and limits the excessive vegetative growth interfering with seed formation (an analytical review)

Nikolay N. FESENKO [1], Zorrida I. GLAZOVA [1], Ivan N. FESENKO [1, 2]

Received May 05, 2020; accepted May 26, 2020.
Delo je prispelo 05. maja 2020, sprejeto 26. maja 2020.

Cold stress at seedlings stage of buckwheat optimizes development of both roots and aboveground biomass and limits the excessive vegetative growth interfering with seed formation (an analytical review)

Abstract: Buckwheat, sown in warmed soil, quickly sprouts, grows and successfully competes with annual weeds. However, such agronomic practice does not always lead to a good grain yield, because a powerful, abundantly flowering buckwheat plants often give only a minimal amount of seeds due to a lack of moisture in the upper layer of soil during flowering. This behavior of buckwheat is described as "overgrowth (with poor seed formation)". This phenomenon is caused by the relative weakness of the root system of this species: the specific feature of buckwheat is a very fast transition to formation of secondary roots, which gives advantages at the first stages of development, but restricts the roots growth later. Buckwheat flowering is stretched in time. First half of this developmental stage which is most important for the grain yield is coincided with the most intensive vegetative growth. The lowered temperature at the seedlings stages slows the growth of the aboveground biomass and accelerates the growth of the roots that at later stages favorably affects the conditions for seeds development and, respectively, the grain yield. It explains why early sowing time is favorable for the grain yield of buckwheat.

Key words: buckwheat; overgrowth; sowing date; grain yield

Hladni stres na razvojni stopnji sejanke navadne ajde optimizira razvoj korenin in nadzemne biomase in omejuje prekomerno vegetativno rast, ki zavira tvorbo semen (analitični pregled)

Izvleček: Navadna ajda, posejana v topla tla hitro vzkali in hitro raste in uspešno tekmuje z enoletnimi pleveli.Vendar pa takšna agronomska praksa ne vodi vedno k dobremu pridelku zrnja, ker daje močno in obilno cvetenje ajde pogosto le minimalno količino semen zaradi pomanjkanja vode v zgornjih plasteh tal v času cvetenja. To obnašanje ajde je opisano kot "pretirana rast (s šibko tvorbo semen).Ta pojav povzroča relativna šibkost koreninskega sistema te vrste, katerega posebnost je zelo hiter prehod k tvorbi sekundarnih korenin, kar daje prednost v začetnem razvoju, a omejuje rast korenin kasneje. Cvetenje navadne ajde je časovno raztegnjeno. Prva polovica tega razvojnega obdobja, ki je najpomembnejša za pridelek zrnja soupada z najmočnejšo vegetativno rastjo. Znižanje temperature na razvojni stopnji sejanke upočasni rast nadzemnega dela in pospeši rast korenin, kar v kasnejših fazah razvoja ugodno vpliva na razmere za razvoj semen in s tem na pridelek zrnja. To razloži zakaj je zgodnja setev navadne ajde ugodna za pridelek zrnja.

Ključne besede: navadna ajda; pretirana rast; čas setve; pridelek zrnja

---

[1] Federal Scientific Center of Grain Legumes and Groats Crops, 302502, p/o Streletskoe, Orel, Russia
[2] Corresponding author, e-mail: ivanfesenko@rambler.ru

1 INTRODUCTION

Common buckwheat, *Fagopyrum esculentum* Moench., is a nectariferous plant with prolonged flowering (usually up to harvesting), which is cultivated in many countries of the northern hemisphere as a grain crop, appreciated for the excellent dietary and healing properties of products from its grain. Maintenance of obligate cross-pollination in plants is usually associated with significant energy costs (Solbrig, 1979; Cruden & Lyon, 1985). In buckwheat, the attraction of pollinators (mainly bees) is provided by excessively abundant, showing flowering. More than 90% of the flowers do not form seeds and die off (buckwheat flower lives 1 day). Such or close to this manner of flowering and seed formation is quite typical for buckwheat and can be considered the norm. Sometimes seed formation could be increased, and some optimists found it possible to talk about a very high potential for buckwheat grain productivity (> 10 t ha^{-1}, if most of the flowers give seeds). However, in buckwheat, the period of the beginning of flowering is overlapped with the most intensive vegetative growth, and under unfavorable conditions (for example, drought), the vegetative growth has an advantage over seed formation, which leads to the mass death of emerging ovaries (Fesenko, 1983). At the same time, the advantage of vegetative growth can present itself even more clearly in very favorable conditions of abundance of moisture and mineral nitrogen in the soil, when the powerful coenosis with abundant flowering doesn't exhibit the appropriate formation of seeds. Such behavior of buckwheat in Russian agronomic jargon is described by the term "zhirovanie" (the approximate translation is "obesity", but the common sense is the "overgrowth with poor seed formation" or, shorter, the "overgrowth"). This is the most distinct for powerful, i.e. later-ripening buckwheat varieties. In this context, buckwheat had a reputation of a "mysterious" crop that could give an acceptable yield on poor soil and, on the contrary, on rich soil the yield could be poor despite the powerful growth and abundant flowering.

There are fairly reliable guesses about the nature of this phenomenon (Fesenko, 1983), however, it was not possible to do special experiments revealing the physiological mechanism of "the overgrowth", and this article became possible only as a summary of various sources, the reason for collecting which was the impressions of first author when visiting the agrotechnical experiment studying the sowing time of the variety Ballada in 1983. This variety, quite late ripening and powerful, turned out to be a "model" for studying the "overgrowth", and the year 1983 was a provocative for a clear manifestation of this phenomenon (unusually early, warm and humid spring and hot, moderately moistened summer). This experiment was conducted during several years (Shashkin & Mazalov, 1992), but only in 1983 the "overgrowth" proved to be a full-fledged qualitative trait, whereas in other years of this work, as well as in other cited works with accents to sowing dates, this phenomenon could be recognized only by quantitative differences in grain yield.

Trying to identify the physiological mechanism so strongly affecting the buckwheat grain yield we found it necessary to do this in context of the specific climatic conditions of the northern farming systems which influenced the results of cited works.

The "line" limiting agriculture from the North crosses the entire territory of Russia and can be prolonged through Japan (Hokkaido) and Canada where buckwheat is also cultivated. The border status is associated with the concept of "risk farming zone". The main limiting factor here is a short frost-free period, which often complicates the formation of soil with good characteristics. This can restrict the yield of main grain crops. Under such conditions, buckwheat can be quite competitive as a relatively unpretentious crop.

Frost-resistant spring cereals are sown as soon as possible, sometimes almost immediately after snowmelt, to avoid mass diseases and insects damages. Frost-sensitive buckwheat is the later crop, which is sown a month later, when nightly frosts possibility becomes minimal.

It allows carry out the effective eliminating weeds' seedlings by mechanical soil cultivation before sowing. Buckwheat seeds sown in a well-warmed soil prepared in this way quickly germinate, and the young plants are able to quickly cover the threadlike seedlings of annual weeds. These are the criteria for an optimal sowing period. But how does this affect the grain harvest? This is the topic of our review, where we collected the observations of buckwheat made since the start of breeding work with it in the Orel region and other regions of the world, which together provide an opportunity to explain the features of its yield' reactions to growing conditions. In the article is also mentioned Tartary buckwheat, *F. tataricum* Gaertn, a self-pollinated species, which at present time has a prospect to be introduced into Russian agriculture.

2 OREL REGION (RUSSIA), 53° N

This region is considered favorable for the cultivation of main crops, including buckwheat. Here we can use the written and personal testimonies of researchers over the past century, during which revolutionary changes occurred in the energy saturation of agriculture and, accordingly, in standard agricultural technology.

In the period before the widespread of agricultural machinery, plowing of the fields was carried out mainly by horse-drawn wooden plough, that is, without furrow slice inversion. This led: 1) to delay spring plowing and sowing of early spring crops, which is why large areas were sown with late spring crops (buckwheat, millet); 2) to accumulation of weeds' rhizomes. In the fields with the most weediness after snow melting spring grazing of cattle was carried out, and plowing and sowing of buckwheat were conducted in June (Krotov, 1960).

Local buckwheat was of an early maturing type, but, apparently, these populations were quite heterogeneous in a number of properties, and on their basis as result of long-term (from 1901 to 1909) earlier (May) sowing and machine sorting of seeds a more powerful and large-grained mid-ripening variety Bogatyr was developed at the Shatilov's Research Station (Fesenko, 1990).

After widespread of machine agriculture, autumn plowing, as well as early spring plowing became the norm, that allowed for timely spring sowing on unlimited areas. It sharply reduced the pest damage to early spring seedlings, and barley, having the opportunity to "get away" from the mass defeat by the *Oscinella* sp., became the most technologically advanced and productive spring crop. As a result, late spring crops began to decline fast. But the "atomic era" has reversed this process, as in Russia buckwheat has become mandatory when planning sowing areas.

The machines era fixed the optimal timing of sowing buckwheat in Orel region for the third decade of May for more than half a century. It was confirmed in agrotechnical experiments (Losev & Khlebnikov, 1973). Earlier sowing was considered impractical due to the higher probability of night frosts and the lack of time for pre-sowing weed control (Losev & Khlebnikov, 1973).

The weather dynamics in the spring months follow a standard scenario, but significant variation is possible between years. At the beginning of the 1980s, earlier and warmer springs became frequent in the Orel region, which is probably consistent with the syndrome of global warming. In those years, the system of regional experimental agricultural institutions has actively working in the USSR. New types' varieties of buckwheat were created in Institute of Grain Legumes and Groats Crops. It required studying varietal agricultural technology, including experiments with sowing dates. In 1983-1991 such experiments were carried out with new mid-ripening buckwheat variety Ballada (Shashkin & Mazalov, 1992) which was sown in the following dates: 1) May 8-11; 2) May 15-16; 3) May 24-26 (standard); 4) June 5-6 and 5) June 9-11. In terms of grain yield depending on the sowing dates next results were obtained (average and range): 1) 1.89 (1.49 ... 2.60) t ha^{-1}; 2) 1.68 (1.07 ... 2.34) t ha^{-1}; 3) 1.51 (0.37 ... 2.02) t ha^{-1}; 4) 0.88 (0.09 ... 1.96) t ha^{-1}; 5) 0.54 (0.06 ... 1.22) t ha^{-1}.

Thus, the maximum yield was obtained at the first (early) term of sowing, the following periods were characterized first by a smooth (terms 2 and 3), and then by a sharp (terms 4 and 5) decline in grain yield with the excellent appearance of these plots. The authors explained the result by the fact that the first sowing period allowed the Ballada cultivar 6 years out of 9 catches the most favorable conditions in the first half of the flowering period which was critical for buckwheat (Shashkin & Mazalov, 1992). In parallel experiment with a new early ripening variety Skorospelaya 86, the largest grain yield was obtained after sowing on May 14–16 and May 24–26, which was explained from the same standpoint (Shashkin & Mazalov, 1992).

3 HOKKAIDO (JAPAN), 43-44° N

The island is located far south of Orel, however, the surrounding sea is cold and agricultural development of the island has occurred recently, i.e. to the beginning of the 20th century. The optimal time for sowing buckwheat here is considered the beginning of June. Later sowing is also practiced (Honda et al., 2010).

The paper by Honda et al.(2010) presents data for 5 years (2004 ... 2008) obtained for three varieties that were sown in three terms: mid-May (early), early June (standard) and the end of June (late). The varieties under the study were different in morphological type: 'Kitawasesoba' is the large-grained standard, 'Kitayuki' is the late-ripening small-grained variety, and 'Kitanomashu' is the new determinant variety.

The varieties showed quite noticeable differences in grain productivity (Kitanomashu> Kitawasesoba> Kitayuki), but a very similar reaction both to the sowing date and to the conditions of the year. All three varieties gave the maximal average (for five years) grain yield after early sowing: three years out of the five all the varieties manifested better results at early sowing compared to sowing in "optimal period". The authors concluded that the advantage of early sowing was due to the best weather conditions during the flowering period, which 3 years out of five was provided by early sowing. The authors also cited the Canadian (Gubbels, 1977) and Japanese works in which similar results were obtained.

4 DISCUSSION

There is a relationship between genetically determined traits of root and crop productivity (Kell, 2011; Narayanan et al., 2014). Since the root system is a dynamic formation that is influenced by environmental factors (Lynch, 1995; Grossman & Rice, 2012), the success of breeding changes in the root system in crops depends not only on a specific trait and the nature of its inheritance, but also on the use of a particular farming system together with soil characteristics (Botwright Acuna & Wade, 2012; Meister et al., 2014).

Buckwheat belongs to the group of plants in which the initiation and development of primordia of the lateral roots occurs in the apical meristem of the main root (O'Dell & Foard, 1969). In addition, due to the initiation of the groups of primordia of lateral roots in embryogenesis, these species are characterized by early branching of the main root after germination (Dubrovsky & Laskowski, 2017). The rapid development of the root system allows compete with representatives of other species for soil resources and quickly gain significant biomass (Ilyina et al., 2017).

At the same time, buckwheat is much inferior to the grain cereals in the final development of the root system (Hagiwara et al., 1998), surpassing them in absorption capacity of roots (Fesenko, 1983). Therefore, the relative weakness of the buckwheat root system may be the cause of the instability of its grain productivity. The effect of the overgrowth with poor seed formation is especially explicit, when buckwheat, after sowing under favorable conditions, develops a powerful above-ground vegetative mass, actively blossoms, but gives a low grain yield.

From this point of view, the experiments by Park et al. (2010), who were optimizing the process of buckwheat seeds germination to obtain seedlings for early spring salad, are interesting. Seeds (30 g) were washed and soaked at a temperature of 25 °C. The seeds that started to germinate were divided into 3 fractions of 10 g each and placed on the steel tray with double layer steel net. The seedlings were grown in the dark under optimal moisture conditions and at stable temperature of 20 °C, 25 °C, and 30 °C, respectively. On the 9th day of such cultivation, the seedling yield was weighed and the length of their roots was measured.

In common buckwheat *F. esculentum*, the seedling yield was the smallest at 20 °C (1.66 g) and increased sharply with increasing temperature to 25 °C (2.73 g) and, especially, to 30 °C (3.30 g). At the same time, the root length was maximal at 20 °C (5.93 cm), with increasing temperature it decreased to 4.80 cm at 25 °C and to 5.23 cm at 30 °C.

This experiment did not aim to determine the root availability of the obtained plants depending on the temperature of cultivation, however, we can use its results for a comparative description of the share of roots in the obtained plant biomass, because the mass of seeds in the studied variants was the same (10 g), but the mass of seedlings varied sharply. The ratio of root length to biomass was 5.93 cm: 1.66 g = 3.57 cm g^{-1} at 20 °C, 4.80 cm: 2.73 g = 1.76 cm g^{-1} at 25 °C (i.e. 2 times less than at 20 °C) and 5.23 cm: 3.30 g = 1.59 cm g^{-1} at 30 °C (i.e. 2.2 times less than at 20 °C).

Thus, at the earliest stage of plant development under optimal growth conditions, the misbalance was initiated, which after beginning of flowering can sharply negatively affect the formation of the grain yield as the fast growth of the aboveground biomass was not sufficiently supported by a more slowly growing root system.

A similar trend could be observed when *F. tataricum* seeds were germinating, but taking into account their smaller size (the conditions were the same)(Park et al., 2010). The seedling yield here was 1.06 g at 20 °C, 1.86 g at 25 °C and 1.90 g at 30 °C. In this case, the length of the roots differed slightly: 4.86 cm, 4.90 cm and 5.13 cm, respectively. The biomass yield at 25 °C increased 1.75 times compared to 20 °C, and the ratio "root : biomass" decreased, respectively, from 4.58 cm g^{-1} (4.86 cm : 1.06 g) to 2.63 cm g^{-1} (4.90 cm : 1.86 g). These indicators are higher than those of *F. esculentum*, however, the tendency was clearly manifested: higher temperature leads to an increase in the aboveground biomass with a decrease in its root support.

In our analysis of this experiment, the categories "more/less" were used, i.e. here we begin to consider the overgrowth with poor seed formation as a quantitative trait. This allows us to include in the analysis not only extreme cases, but also almost the entire set of facts obtained during the experimental and practical cultivation of buckwheat. Such an approach makes it possible to distinguish between the level of imbalance between the power of the entire biomass of the plant and its root support, and evaluate this trend for any variety.

Powerful mid-ripening and late-ripening varieties are usually prone to the overgrowth with poor seed formation under favorable conditions including fertilized soil, while rapidly blooming early ripening varieties are genetically more resistant to this phenomenon. So, the early-ripening variety Skorospelaya 86 manifested the best result when sowing date was standard, but the mid-ripening variety Ballada, prone to strong growth, shown best yield when sowing in the earliest terms (Shashkin & Mazalov, 1992), i.e. when temperature was lower. In addition, the Ballada variety gave the smallest sum of yields in the five sowing terms in 1983, when the most favorable combination of early heat and moisture supply was being. That year, at early sowing date, the yield was only 1.56 t ha^{-1}, in contrast with the perfect characteristics of the cenosis in all other aspects, the second sowing term showed 1.12 t ha^{-1}, and the yield of the next three periods was 0.37, 0.09 and 0.06 t ha^{-1} also in spite of perfect characteristics of the fields, i.e. the proportions of their vegetative development and grain yield more and more clearly consistent with the concept of excessive vegetative growth with poor seed formation. The maximal yield was experimentally obtained in a rather cool 1990 (2.6 t ha^{-1} with early sowing) (Shashkin & Mazalov, 1992). The variety Ballada is able to form a larger biomass than the Bogatyr and Krasnostreletskaya varieties, which showed the best result when sowing in the third decade of May 18 years before (Losev & Khlebnikov, 1973).

Japanese varieties are sensitive to light day length (short-day type) and exhibit enhanced branching in Hokkaido (Fesenko et al., 1999), i.e. form a powerful, even excessive biomass. According to the cited publication (Honda et al., 2010), the alteration of this genotype into a determinant type of growth was very effective, because a new determinant variety has proven itself as the most productive. In this work, it was noted that early-sown plants were shorter than those sown in June. The authors concluded that the increase in yield in the first term was associated with increased resistance to lodging (Honda et al., 2010). At the same time, it can be a consequence of both the reaction of the short-day varieties on a shorter day in May, and a heat deficiency at this time. However, a decrease in above-ground biomass can be functionally associated with better root support of the flowering and seed formation processes. It is necessary to note the best root support of the seed filling process is one of the advantages of determinant buckwheat varieties over ones with indeterminate growth habit (Martynenko, 1988).

An earlier sowing period is usually associated with a greater likelihood of night frost. Not all May night frosts observed in the Orel region were fatal for buckwheat. If the frost was due to cold and wet weather, most often a partial freezing of seedlings was observed, which, however, did not always affect the final result, because the surviving plants were sufficient for the formation of a grain yield in conditions of reduced competition. Interestingly, in the Hokkaido experiment, the maximum grain yield was obtained in 2007, when the seedlings of the first sowing period fell under freezing on May 28 (Honda et al., 2010). Apparently, the moist marine climate played the role of a buffer and did not allow the temperature to drop below a critical level, and the inhibition of the aboveground biomass growth appeared to be optimal for harmonizing the process of seed formation.

Frost together with drought is more dangerous when large daily temperature fluctuations are possible, for example, from +17 °C during the day to -3 °C at the end of the night. The geographical conditions of continental Canada and Siberia suggest a high probability of such severe night frosts in late spring. Canadian varieties are quite late-ripening, and for this reason are prone to form excess biomass when sowing in the frost-free period. This is probably why the Canadian buckwheat breeding program emphasized the development of frost-resistant varieties (Campbell, 2003). Similar work was carried out in Siberia (Kostyro, 1971). But it is too early to talk about the successes of this direction.

Theoretically, it is possible to minimize the effect of the “overgrowth” after sowing in heated soil using effective retardants that reduce the vegetative growth of buckwheat plants. Occasionally, publications appear in which a physiologically active substance is considered as a factor that dramatically increases the yield of buckwheat grain. An example is the article by Szelezniak et al. (2010), which describes the experience of herbicides application on buckwheat crops. Only chemicals to which buckwheat is relatively tolerant have been compared. Sowing was carried out on May 26, 2009, i.e. into warm soil. Interestingly, all seven of the studied chemicals gave an increase in grain yield; three of them gave the increase more than 2 times. Since all these herbicides can be regarded as retardants in terms of the physiological effect on buckwheat, it can be assumed that their use not only reduced competition from weeds, but also optimized the root support of the plant generative sphere during flowering due to reduction of aboveground biomass, also reducing auto-competition in the cenosis.

Unfortunately, the cited work, like many others, does not provide significant information on the physiological effects of the chemicals on plant organs. Our approach based on a quantitative analysis of the “overgrowth” in buckwheat can stimulate such studies. It seems that the level of root development (and final yield) of the buckwheat plant is largely determined at the first stages of growth, which are most easily controlled by duplicating field experiments by hydroponic ones (Park et al., 2010). It is necessary to note that our conclusions are based not only on the cited results of accurate single-factor experiments, but also on many years experience in studying buckwheat from various perspectives (genetics, cultivation technology, breeding), which allows us to form an integral natural-philosophical view on the problem. But while, on the existing assortment of buckwheat, French farmers get the maximum yield in the marine climate of the Brittany province (http://www.fao.org). Unfortunately, we do not have information about the peculiarities of the cultivation technology used there. It can be assumed that the absence of late spring night frosts over the years (the Gulf Stream influence) allows early buckwheat sowing. As result, the seedlings develop under relatively low positive temperatures, which stops the premature excessive vegetative growth of the variety La Harpe widespread there. Therefore, its plants begin to bloom when the root system achieved maximum possible development (relative to the above-ground biomass), sufficient to provide flowering and seed formation, which in this developmental phase is overlapped with intensive vegetative growth.

5 REFERENCES

Botwright Acuna, T.L., Wade L.J. (2012). Genotype × environment interaction for root depth of wheat. *Field Crops Research*, *137*, 117-125. https://doi.org/10.1016/j.fcr.2012.08.004

Campbell, C. (2003). Buckwheat crop improvement. *Fagopyrum*, *20*, 1-6.

Dubrovsky, J.G., Laskowski, M. (2017). Lateral root initiation. In:B. Tomas, B.G. Murray & D.G. Murphy (Eds.) *Encyclopedia of applied plant sciences (2nd edition)*(pp. 256-264). Oxford: Academic Press.

Fesenko, N.N., Martynenko, G.E., Funatsuki, H., Romanova, O.I. (1999). Comparison of some buckwheat cultivars in conditions of Russia and Hokkaido using morphological descriptors-indicators of duration of vegetation period. *Research Bulletin of Hokkaido National Experimental Station*, *№267*, 149-156.

Fesenko, N.V. (1983). *Breeding and seed farming of buckwheat.* Moscow: Kolos. (in Russian)

Fesenko, N.V. (1990). Research and breeding work with buckwheat in USSR (Historic Survey). *Fagopyrum*, *10*, 47-50.

Grossman, J.D., Rice, K.J. (2012). Evolution of root plasticity responses to variation in soil nutrient distribution and concentration. *Evolutionary Applications*, *5*, 850-857. https://doi.org/10.1111/j.1752-4571.2012.00263.x

Gubbels, G.H. (1977). Interaction of cultivars sowing date and sowing rate on lodging, yield and seed weight of buckwheat. *Canadian Journal of Plant Science*, *57*,317-321. https://doi.org/10.4141/cjps77-048

Hagiwara, M., Inoue N., Matano, T. (1998). Allometry among roots, leaves and flower clusters in common buckwheat. *Fagopyrum*, *15*, 29-34.

Honda, Y., Mukasa, Y., Suzuki, T. (2010). Effect of earlier sowing on summer type of common buckwheat in the northern area of Japan. In: V.I. Zotikov, N.V. Parakhin (Eds.) *Proceedings of 11th International Symposium on Buckwheat* (pp. 612-615). Orel: Kartush.

Ilina, E.L., Kiryushkin, A.S., Tsyganov, V.E., Pawlowski, K., Demchenko, K.N. (2017). Molecular, genetic and hormonal outlook in root branching (review). *Sel'skokhozyaistvennaya biologiya [Agricultural Biology]*, *52*, 856-868. https://doi.org/10.15389/agrobiology.2017.5.856eng (in Russian)

Kell, D.B. (2011). Breeding crop plants with deep roots: their role in sustainable carbon, nutrient and water sequestration. *Annals of Botany*, *108*, 407-418. https://doi.org/10.1093/aob/mcr175.

Kostyro, V.P. (1971). Creation of cold-resistant varieties of buckwheat. In: *Breeding, genetics and biology of buckwheat* (pp. 25-28). Orel: VNIIZBK. (in Russian)

Krotov, A.S. (1960). From the history of buckwheat cultivation in the USSR. In: *Materials on the history of agriculture and peasantry of the USSR,* (pp. 414-456). Moscow: USSR Academy of Science. (in Russian)

Losev, S.I., Khlebnikov, A.I. (1973). About the timing of sowing buckwheat. *Bull. NTI VNIIZBK (Orel), №4*, 72-78. (in Russian)

Lynch, J. (1995). Root architecture and plant productivity. *Plant Physiology*, *109*, 7-13. https://doi.org/10.1104/pp.109.1.7

Martynenko, G.E. (1988). Morphobiological features and prospects of breeding of the determinant form of buckwheat. In: *Prospects for increasing buckwheat yield and grain quality* (pp. 21-24). Kishinev. (in Russian)

Meister, R., Rajani, M.S., Ruzicka, D., Schachtman, D.P. (2014). Challenges of modifying root traits in crops for agriculture. *Trends in Plant Science*, *19*, 779-788. https://doi.org/10.1016/j.tplants.2014.08.005

Narayanan, S., Mohan, A., Gill, K.S., Prasad, P.V.V. (2014). Variability of root traits in spring wheat germplasm. *PLoS ONE*, *9*, e100317. https://doi.org/10.1371/journal.pone.0100317.

O'Dell, D.H., Foard, D.E. (1969). Presence of lateral root primordia in the radicle of buckwheat embryos. *Bulletin of the Torrey Botanical Club*, *96*, 1-3. https://doi.org/10.2307/2484002

Park, C.H., Kim, J.H., Chang, K.J., Woo, S.H., Lee, M.H., Briatia, X. (2010). Effect of temperature, deep sea water, and seed quality on growth of buckwheat sprouts. In: V.I. Zotikov, N.V. Parakhin (Eds.) *Proceedings of 11th International Symposiumon Buckwheat* (pp. 631-636). Orel: Kartush.

Shashkin, Yu.A., Mazalov, V.I. (1992). Sowing dates and yield of buckwheat. In:*Improving the breeding and technology of cultivating legumes and groats crops* (pp. 222-225). Orel: VNIIZBK. (in Russian)

Szelezniak, E., Podolska, G., Rubicki, L. (2010). Influence of herbicides on weed control in buckwheat and buckwheat yielding. In V.I. Zotikov, N.V. Parakhin (Eds.) *Proceedings of 11th International Symposium on Buckwheat* (pp. 661-665). Orel: Kartush.

FAO. (2014). FAOSTAT database. Retrieved from http://www.fao.org/faostat/en/#home

doi:10.14720/aas.2020.116.1.1547 Original research article / izvirni znanstveni članek

Effect of sowing date and some organic extracts on organic production of sweet corn

Saad ABOUL-EL-HASSAN[1, 2], Mohamed S.A. EMAM[3], Manal M.H. GAD EL-MOULA[3]

Received February 27, 2020; accepted June 16, 2020.
Delo je prispelo 27. februarja 2020, sprejeto 16. junija 2020

Effect of sowing date and some organic extracts on organic production of sweet corn

Abstract: Field experiment was conducted at the experimental farm of the Central Laboratory for Agricultural Climate, Agricultural Research Center, Giza, Egypt, during two successive seasons of 2017 and 2018. This experiment aims to improve the organic production of sweet corn via some organic extracts under different sowing dates. The seeds of sweet corn (Misthi F1 Hybrid) were planted on three different dates on 15 April, 10 May and 5 June in both seasons. Extracts of compost, vermicompost and chicken manure with applying half dose of compost were compared to full dose of compost. Results showed that early sowing had positive effect on growth, yield and ear properties of sweet corn. Applying half dose of compost with adding vermicompost extract gave the highest growth, yield and ear properties of sweet corn without significant differences compared to 50 % compost + chicken manure extract treatment in plant height and leaves number per plant. The treatments of the half dose of compost with adding any organic extracts were superior to the full dose of compost in all studied traits. This study revealed that early planting (April 15) with applying a half dose of compost + vermicompost extract produced the highest yield and quality of sweet corn ears.

Key words: sweet corn; sowing date; compost extract; vermicompost extract; manure extracts

Učinki datuma setve in izvlečkov nekaterih organskih gnojil na pridelek sladke koruze

Izvleček: Poljski poskus je bil izveden na poskusni postaji Central Laboratory for Agricultural Climate, Agricultural Research Center, Giza, Egypt, v rastnih sezonah 2017 in 2018. Namen poskusa je bil izboljšati ekološko pridelavo sladke koruze z izvlečki nekaterih organskih gnojil in različnimi datumi setve. Semena sladke koruze so bila v obeh rastnih sezonah posejana v treh različnih terminih, 15. aprila, 10. maja in 5, junija. Učinki izvlečkov komposta, vermikomposta in kokošjega gnoja s polovičnim dodatkom komposta so bili primerjani z obravnavanjem s polnim dodatkom komposta. Rezulatati so pokazali, da je imela zgodnja setev pozitivne učinke na rast, pridelek in lastnosti storžev sladke koruze. Uporaba polovičnega odmerka komposta z dodatkom vermikomposta je dala najboljšo rast, največji pridelek in najboljše lastnosti storžev sladke koruze brez značilnih razlik v primerjavi z obravnavanjem s 50 % komposta in izvlečkom kokošjega gnoja glede na višino rastlin in število listov na rastlino. Obravnavanja s polovičnim odmerkom komposta so bila boljša kot tista s polnim odmerkom komposta v vseh preučevanih lastnostih. V raziskavi je bilo ugotovljeno, da je bil dosežen največji pridelek in dosežene najboljše lastnosti storžev sladke koruze pri zgodnji setvi (15. april) in uporabi polovičnega odmerka komposta z dodatkom izvlečka vermikomposta.

Ključne besede: sladka koruza; datum setve; izvleček komposta; izvleček vermikomposta; izvlečki gnojil

---

[1] Central Lab of Organic Agriculture, Agriculture Research Center, Egypt

[2] Corresponding author, e-mail: saad0777@yahoo.com

[3] Central Laboratory for Agricultural Climate, Agricultural Research Center, Egypt

1 INTRODUCTION

Sowing date greatly affects the growth and yield of sweet corn. Either early or late cultivation can result in lower yield since the probability exists that adverse climatic conditions can happen after planting or during the growing season (Nielson et al., 2002). For optimization of yield, planting at the appropriate date is very critical as delay in sowing date can lead to a linear decrease in growth and yield (Anapalli et al., 2005, Namakka et al., 2008, Kamara et al., 2009, Peykarestan and Seify, 2012, Ibrahim et al., 2013, Maga et al., 2015, Rah Khosravani et al., 2017, Kaur and Kaur, 2018). They also indicated that early planting in the spring is optimum and more efficient than delayed planting.

Sweet corn (*Zea mays* L. var. *rugosa*) is one of promising vegetable crops in Egypt for local market and exportation. It is different from other maize crops (field maize and popcorn), where its kernels have high sugar content in the milk or early dough stage. Sweet corn has been widely considered more beneficial than maize production due to the shorter growing season and higher sale price, which reduces production costs and eventually increases farmers' income (Thakur et al., 2009). In addition, its economic values might be double when it is organically grown due to increasing consumer's demands and limited supply of it.

Organic farming products are becoming very essential in today's world to maintain the ecosystem and human health; there is a growing global demand for organic products in the world. The total organic area in the world reached 57.8 million hectares; the cultivated area in Egypt is about 105.9 thousand hectares according to FiBL and IFOAM (2018). Organic agriculture depends on compost as the main source of fertilization. Compost is usually added at once before planting, this leads to the nutrients available from compost insufficient to crop requirements. Consequently, most organic crops need some additives that improve growth and productivity during the growing season (Gross et al., 2008 and Abou-El-Hassan et al., 2014). Some organic extracts (compost, vermicompost and chicken manure extracts) can be used for improving organic production (Lopez-Espinosa et al., 2013; Ibrahim and Ibrahim, 2014; Nawar and Ibraheim, 2014).

Compost is an aerobically decomposed organic material derived from plants and/or animal residues by mesophilic and thermophilic microorganisms (Martens, 2000 and Insam & de Bertoldi, 2007). Vermicompost is product of organic matter degradation through interactions between earthworms and microorganisms (Edwards and Neuhauser, 1988).

Organic extracts are a liquid extracts produced from soaking various kinds of compost or organic manure in water at rate 1 : 5 and is left for a defined period; to create a liquid rich in organic and inorganic soluble nutrients and a large number of beneficial microorganisms (ROU, 2007). Organic extracts became a common agriculture practice in sustainable farming (Gross et al., 2008) as a balanced source of nutrients in available form in the rizosphere, growth stimulant and disease suppressor. In addition to its beneficial impacts on soil physical and chemical attributes as well as soil biodynamic activity. Organic extracts have positive effects on plant growth and soil properties (Abbasi et al., 2002, Biocycle, 2004, Gharib et al., 2008 and Meshref et al., 2010). Many studies indicated that application of organic extracts enhanced the growth, yield and quality for many crops such as Abou-El-Hassan et al. (2002) on cantaloupe, El-Tantawy et al. (2009) on potato, Ghobrial et al. (2009) and Mahmoud (2011) on faba bean, Seran and Shahardeen (2013) on vegetable cowpea, Bulalin et al. and Kovacik et al. (2015) on maize, Rogelio (2017) on sweet corn, Pokhrel et al. (2017) on tomato and Shaheen et al. (2017) on common bean.

Therefore, this study aimed to evaluate possibility of improving the organic production of sweet corn via some organic extracts under different sowing dates.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SITE

Field experiment on sweet corn was carried out at the experimental farm of the Central Laboratory for Agricultural Climate (CLAC), Agricultural Research Center, Giza, Egypt, during two successive seasons of 2017 and 2018.

2.2 PLANT MATERIAL

Seeds of sweet corn ('Misthi F1' hybrid) were sown in the field on three different dates on 15 April, 10 May and 5 June in the both seasons.

2.3 SOIL PROPERTIES

The experiment was conducted in clay soil using drip irrigation system, the soil was analyzed according to FAO (1980) and the results are tabulated in Table 1. Weather data in the experimental region as air temperature and relative humidity (RH %) were recorded by the

Table 1: Physical and chemical properties of the experimental soil

Clay %	Silt %	Sand %	Texture	pH	EC dS m^{-1}	Cations meq^{-1} Ca^{++}	Mg^{++}	Na^{+}	K^{+}	Anions meq^{-1} HCO_3^-	Cl^-	$SO_4^=$
48.76	40.91	10.33	Silte clay	7.87	1.03	2.32	1.14	5.33	1.52	2.27	4.84	3.13

Table 2: Weekly average of air temperature and relative humidity in Dokki region during growing seasons

		First season (2017)						Second season (2018)					
		Air temperature [°C]			Relative humidity [%]			Air temperature [°C]			Relative humidity [%]		
Months	Weeks	aver	min	max	aver	min	max	aver	min	max	aver	min	max
April	1st	20.4	14.8	27.3	55.3	26.4	85.7	20.7	14.8	27.2	55.5	27.4	84.3
	2ed	21.5	16.6	26.7	52.3	31.7	82.0	21.1	15.9	26.5	52.9	32.1	79.6
	3rd	26.1	18.9	31.0	34.5	15.1	62.7	26.1	18.9	31.0	34.5	15.1	62.7
	4th	23.9	18.1	29.2	38.5	21.2	66.6	24.9	18.8	30.2	36.6	20.1	63.7
May	1st	26.1	20.4	30.5	44.1	26.9	74.2	26.7	21.0	33.3	48.9	27.3	75.8
	2ed	31.1	23.5	36.5	28.9	15.3	56.5	25.5	19.1	32.3	46.8	24.6	76.6
	3rd	28.5	22.9	33.3	36.0	20.0	62.9	28.3	21.5	35.9	43.0	19.2	70.9
	4th	30.1	24.7	34.3	29.5	17.5	51.0	29.3	23.5	36.0	44.6	23.7	68.0
June	1st	31.5	26.2	35.8	33.4	20.2	55.9	27.3	21.8	33.7	51.4	27.7	78.7
	2ed	30.8	25.3	35.4	39.9	24.6	63.9	29.9	22.6	37.6	46.9	22.2	80.1
	3rd	30.9	25.7	35.7	41.9	24.4	65.9	30.9	25.7	35.7	41.9	24.4	65.9
	4th	30.0	24.2	35.7	47.6	24.9	76.8	30.0	24.2	35.7	47.6	24.9	76.8
July	1st	32.9	27.5	37.6	45.3	27.0	71.7	30.1	24.6	36.7	55.1	27.2	80.6
	2ed	31.7	25.7	37.4	46.6	22.4	77.9	29.9	24.9	36.1	55.4	29.1	79.7
	3rd	32.7	27.2	37.5	43.8	23.4	70.4	29.9	24.5	36.7	56.1	30.3	80.5
	4th	31.1	26.3	36.1	51.5	30.5	74.0	30.8	25.2	37.2	53.3	28.8	78.0
August	1st	32.4	27.8	36.7	47.4	28.1	72.0	30.7	25.9	36.8	56.9	31.1	80.2
	2ed	32.2	28.1	36.5	52.8	36.9	72.9	30.3	25.1	36.5	56.0	28.5	81.7
	3rd	31.8	27.1	36.4	45.9	28.0	70.9	29.8	25.1	35.7	55.5	30.6	78.4
	4th	30.5	26.2	34.9	48.9	30.3	70.2	29.6	25.0	35.1	57.4	33.3	79.2

meteorological station of CLAC during two growing seasons and the data are presented in Table 2.

2.4 EXPERIMENTAL PLAN

The soil of the experiment was ploughed and divided into ridges; each plot included three ridges of 60 cm width and 3 m length. The space between plants was 25 cm on one side of ridge.

The treatments included three sowing dates (15 April, 10 May and 5 June) and four fertilizer treatments as follow:

1- Full dose of compost (100 % C) as control

2- Half dose of compost (50 % C) + compost extract (CE)

3- Half dose of compost (50 % C) + vermicompost extract (VE)

4- Half dose of compost (50 % C) + chicken manure extract (ChE)

The design of experiment was split plots with three replicates, where the three sowing dates distributed in the main plots and the four fertilizer treatments were randomly arranged in the sub plots. The plot area was 6 m^2 (3 m length and 2 m width).

Full and half doses of compost were calculated based on nitrogen requirement for sweet corn plants (80 kg/ feddan); that were 8.4 and 4.2 tons /feddan (feddan = 0.42 hectare) respectively. These quantities of compost were added as one dose during soil preparation.

The stock solution of compost extract was prepared

Table 3: The properties of compost, vermicompost and chicken manure

Item	Compost	Vermicompost	Chicken manure
Density (%)	0.77	0.86	0.54
Mositure (%)	18	22	10
pH 1:10	8.43	8.62	8.82
Ec (ds/m) 1:10	4.09	6.34	5.3
OM (%)	28.39	32.03	43.32
OC (%)	17.32	19.54	26.43
Ash (%)	81.61	77.98	56.68
C/N ratio	18.2	12.6	13.3
Total N (%)	0.95	1.55	1.98
N-NH_4 (ppm)	129	48	250
N-NO_3 (ppm)	65	157	45
Total P (%)	0.63	2.22	2.76
Total K (%)	1.18	1.90	1.57

by soaking 10 l compost in 50 l of water without chlorine for two days and was filtrated by plastic net, the clear stock solution was diluted by water without chlorine at rate 1 : 10 according to El-Shinawy et al. (1999) and Abou-El-Hassan (2010). The same procedures were followed to prepare extracts of vermicompost and chicken manure. An air composting process was conducted on the chicken manure for three weeks before use. The properties of compost, vermicompost and chicken manure used in preparation of extracts are illustrated in Table 3. All organic extracts were applied to the soil surface weekly at a rate of 1l m^{-2} beginning after two weeks of planting for two months.

2.5 DATA RECORDED

Sweet corn ears were harvested at early dough stage after 75 to 80 days from planting and total yield was recorded for each plot. Five plants were randomly taken from each plot for measuring plant growth characteristics that included plant height from soil surface to the highest point of the plant, number of leaves per plant, fresh mass of plant and stalk diameter, as well as chlorophyll reading in the fourth upper leaf was recorded by using Minolta Chlorophyll Meter SPAD 501. Nutrient content of N, P and K in sweet corn plants were determined in the fourth upper leaf according to Cottenie et al. (1982). Total nitrogen, phosphorus and potassium were determined by Micro Kjeldahl, spectrophotometer and flame photometer on respectively according to FAO (1980). Ten ears from each plot were taken randomly at harvest to measure ear parameters of mass, length and diameter at central ear. Total soluble solid (TSS) was measured using digital refractometer and total sugars were determined in fresh kernels according to AOAC (2005). Dry matter percent was determined in 100g of fresh kernels.

2.6 STATISTICAL ANALYSIS

Data of the two seasons were arranged and statistically analyzed by the analysis of variances according to Snedecor and Cochran (1980) with SAS software, version 2004. Treatment means were compared using Tukey test at significance level 0.05.

3 RESULTS AND DISCUSSION

3.1 GROWTH CHARACTERISTICS

The growth characteristics for sweet corn plants under different sowing dates and fertilizers were presented in Tables 4 and 5. Data in both seasons revealed that the highest values of growth characteristics were obtained by sowing on 15 April followed by 10 May, while the sowing on 5 June gave the lowest values. This result may be due to the weather conditions in the early sowing date were the most favorable to sweet corn growth more than other sowing dates as reported by Anapalli et al. (2005), Namakka et al. (2008), Kamara et al, (2009), Peykarestan and Seify (2012) and Ibrahim et al. (2013).

The treatment of 50 % compost + vermicompost extract had the maximum growth characteristics of sweet corn plants compared to other treatments in both sea-

Table 4: Effect of sowing dates and organic extracts on vegetative growth characteristics of sweet corn plants during 2017 and 2018 seasons

Fertilizer treatments	Plant height (cm)							
	First season				Second season			
	Sowing dates							
	15-Apr	10-May	5-Jun	Mean	15-Apr	10-May	5-Jun	Mean
100 % C	205.3 f	198.7 f	189.7 g	197.9 C	210.3 fg	202.7 g	192.7 h	201.9 C
50 % C + CE	221.3 cde	218.7 de	214.7 e	218.2 B	224.3 cde	221.0 de	218.3 ef	221.2 B
50 % C + VE	235.0 a	227.7 bc	225.0 cd	229.2 A	239.3 a	230.7 bc	228.0 cd	232.7 A
50 % C + ChE	232.3 ab	224.7 cd	223.3 c	226.8 A	237.0 ab	226.7 cd	225.0 cde	229.6 A
Mean	223.5 A	217.4 B	213.2 C		227.7 A	220.3 B	216.0 C	
	Leaf number/plant							
100 % C	14.17 ef	13.83 f	12.67 g	13.56 C	14.67 d	14.50 d	13.33 e	14.17 C
50 % C + CE	16.00 b	15.00 cde	14.33 def	15.11 B	16.00 bc	15.00 cd	14.67 d	15.22 B
50 % C + VE	17.67 a	16.00 b	15.50 bc	16.39 A	17.67 a	16.17 b	15.83 bc	16.56A
50 % C + ChE	17.00 a	15.83 bc	15.17 bcd	16.00 A	17.33 a	16.00 bc	15.50 bcd	16.28A
Mean	16.21 A	15.17 B	14.42 C		16.42 A	15.42 B	14.83 C	
	Stalk diameter (cm)							
100 % C	3.04 de	2.97 e	2.70 f	2.90 D	3.11 ef	2.98 f	2.77 g	2.95 D
50 % C + CE	3.30 c	3.15 d	3.02 de	3.16 C	3.32 cd	3.19 de	3.09 ef	3.20 C
50 % C + VE	3.62 a	3.44 b	3.38 bc	3.48 A	3.65 a	3.47 b	3.40 bc	3.51 A
50 % C + ChE	3.36 bc	3.40 bc	3.37 bc	3.38 B	3.41 bc	3.40 bc	3.39 bc	3.40 B
Mean	3.33 A	3.24 B	3.12 C		3.37 A	3.26 B	3.16 C	

Means followed in same column by similar letters are not statistically different at 0.05 level according to Tukey test.
C = Compost; CE = Compost extract; VE = Vermicompost extract; ChE = Chicken manure extract

Table 5: Effect of sowing dates and organic extracts on vegetative growth characteristics of sweet corn plants during 2017 and 2018 seasons

Fertilizer treatments	Plant fresh mass (g)							
	First season				Second season			
	Sowing dates							
	15-Apr	10-May	5-Jun	Mean	15-Apr	10-May	5-Jun	Mean
100 % C	1.02 e	0.95 f	0.94 f	0.97 D	1.05 e	0.96 f	0.92 f	0.98 D
50 % C + CE	1.16 c	1.08 d	1.04 e	1.09 C	1.17 d	1.09 e	1.05 e	1.10 C
50 % C + VE	1.34 a	1.26 b	1.23 b	1.28 A	1.34 a	1.26 b	1.24 bc	1.28 A
50 % C + ChE	1.25 b	1.19c	1.16 c	1.20 B	1.26 b	1.19 cd	1.16 d	1.21 B
Mean	1.19 A	1.12 B	1.09 C		1.21 A	1.12 B	1.09 C	
	Chlorophyll reading (SPAD)							
100 % C	45.17 ef	45.83 e	42.41 f	44.47 D	49.67 f	49.67 f	46.00 g	48.44 D
50 % C + CE	52.17 cd	50.67 d	49.84 d	50.89 C	57.33 cd	56.67 de	54.00 e	56.00 C
50 % C + VE	57.33 a	55.03 ab	54.18 bc	55.51 A	64.33 a	61.00 b	60.33 bc	61.89 A
50 % C + ChE	54.33 bc	53.89bc	52.17 cd	53.47 B	61.33 ab	60.67 b	58.67 bcd	60.22 B
Mean	52.25A	51.36 A	49.65 B		58.17 A	57.00 A	54.75 B	

Means followed in same column by similar letters are not statistically different at 0.05 level according to Tukey test.
C = Compost; CE = Compost extract; VE = Vermicompost extract; ChE = Chicken manure extract

sons. No significant differences between the treatments of 50 % compost + vermicompost extract and 50 % compost + chicken manure extract in properties of plant height and leaf number per plant. The lowest values in all growth characteristics of plants were resulted from 100 % compost treatment. Improvement in the growth of sweet corn plants with treatment of 50 % compost + vermicompost extract may be due to the role of this extract in the development of the root and enhanced the vegetable growth of plants. Where, vermicompost extract contains high levels of beneficial microorganism, soluble nutrients, organic acids and soluble growth regulators (Keeling et al., 2003; Edwards et al., 2006; Arancon et al., 2007). These results are confirmed with those obtained by Rogelio (2017). He reported that vermicompost extract could be used as growth enhancers of sweet corn, which improved the vegetative growth of plants in terms of stalk diameter, plant height and number of leaves per plant.

The interaction between sowing dates and organic extracts had significant effect on vegetative growth characteristics in both seasons. The highest values of all vegetative growth parameters were recorded by using 50 % compost + vermicompost extract with sowing on 15 April. The lowest vegetative growth parameters were obtained by 100 % compost treatment with sowing on 5 June.

3.2 NUTRITIONAL STATUS

Data in Table (6) indicated that sowing on 15 April in both seasons gave the highest nutrient content of N, P and K in sweet corn leaves without significant differences with sowing on 10 May in N and K content of leaves. Sowing on 5 June gave the lowest N, P and K content. The low nutrient content associated with late planting may be attributed to shortening the vegetable growth period, leading to less vegetable growth (Tables 4 and 5) and lower plant capacity to absorb nutrients. These results confirmed with those reported by Ibrahim et al. (2013) and Rah Khosravani et al. (2017).

The highest concentrations of N, P and K were found in plants that treated by 50 % compost + vermicompost extract without significant differences with 50 % compost + chicken manure extract treatment in N

Table 6: Effect of sowing dates and organic extracts on nutritional status of sweet corn plants during 2017 and 2018 seasons

Fertilizer treatments	N (%)							
	First season				Second season			
	Sowing dates							
	15-Apr	10-May	5-Jun	Mean	15-Apr	10-May	5-Jun	Mean
100 % C	1.469 g	1.447 g	1.317 h	1.411 C	1.487 f	1.470 f	1.322 g	1.426 C
50 % C + CE	1.772 e	1.743 e	1.637 f	1.717 B	1.781 e	1.757 e	1.549 f	1.695 B
50 % C + VE	2.591 a	2.477 bc	2.377 cd	2.481 A	2.593 a	2.493 b	2.389 cd	2.492 A
50 % C + ChE	2.507 ab	2.453 bcd	2.367 d	2.442 A	2.527 ab	2.480 bc	2.363 d	2.457 A
Mean	2.085 A	2.030 A	1.924 B		2.097 A	2.050 A	1.906 B	
	P (%)							
100 % C	0.228 f	0.223 f	0.205 g	0.219 D	0.237 e	0.217 fg	1.247 g	0.567 D
50 % C + CE	0.255 d	0.240 e	0.230 f	0.242 C	0.268 d	0.243 e	0.229 ef	0.246 C
50 % C + VE	0.378 a	0.358 b	0.348 bc	0.361 A	0.382 a	0.362 b	0.345 bc	0.363 A
50 % C + ChE	0.358 b	0.353 b	0.343 c	0.351 B	0.360 b	0.356 bc	0.339 c	0.352 B
Mean	0.304 A	0.294 B	0.282 C		0.312 A	0.295 B	0.540 C	
	K (%)							
100 % C	1.333 g	1.333 g	1.222 h	1.296 D	1.367 g	1.348 g	1.222 h	1.312 D
50 % C + CE	1.740 e	1.699 ef	1.625 f	1.688 C	1.757 e	1.724 e	1.638 f	1.706 C
50 % C + VE	2.456 a	2.409 ab	2.370 bc	2.412 A	2.462 a	2.427 ab	2.383 bc	2.424 A
50 % C + ChE	2.299 cd	2.296 cd	2.272 d	2.289 B	2.333 cd	2.321 cd	2.285 d	2.313 B
Mean	1.957 A	1.934 A	1.872 B		1.979 A	1.955 A	1.882 B	

Means followed in same column by similar letters are not statistically different at 0.05 level according to Tukey test.
C = Compost; CE = Compost extract; VE = Vermicompost extract; ChE = Chicken manure extract

content of leaves. While the lowest concentrations of N, P and K in plants were obtained by 100 % compost treatment. The superiority of vermicompost extract on compost extract might be due to that compost is higher in ammonium content, while the vermicompost is higher in nitrate content, which is the more available form for plant absorption. Besides, the nutrients are released from vermicompost during short time compared to compost and chicken manure. These results are in harmony with those obtained by Pant et al. (2011), Seran and Shahardeen (2013), Bulalin et al. (2015), Kovacik et al. (2015) and Shaheen et al. (2017).

Regarding the interaction between sowing dates and organic extracts, sowing on 15 April with using 50 % compost + vermicompost extract gave the highest N, P and K content of leaves. Sowing on 5 June with applying 100 % compost gave the lowest nutrient content.

3.3 YIELD AND EAR CHARACTERISTICS

Data illustrated in Tables 7 and 8 mentioned that sowing on 15 April produced the highest values of yield and ear characteristics of sweet corn compared to other sowing dates in the two seasons. The lowest yield was produced by sowing on 5 June. Low yield and ear characteristics in the delayed sowing is often due to unfavorable environmental effects such as high temperature, which has adverse effects like increase respiration and transpiration rates thus limit the accumulation of photosynthesis products, which reflected in the low yield and ear quality (Maga et al., 2015 Kaur and Kaur, 2018).

Application of 50 % compost + vermicompost extract produced the highest yield per plant and plot as well as gave the best ear characteristics compared to other treatments. The treatment of 50 % compost + chicken manure extract came in second order, whereas the treatment of 100 % compost gave the lowest values of yield and ear characteristics. The superior vermicompost extract on other extracts and compost only can be attributed to its superiority in stimulating vegetable growth of plants. Where, this treatment improved of growth characteristics as shown in Table 3 and 4, which led to an increase in the process of photosynthesis and better carbohydrate build up, thus improved yield and ear parameters of sweet corn. These results are consistent with those obtained by Bulalin et al. (2015), Kovacik et al. (2015) and Rogelio (2017). They revealed that vermicompost extract when used for nutrition of maize plants significantly improved the growth, yield and quality of corn ears. This is due to the presence of plant growth promoters and its ability to improve the soil condition in rhizosphere zone.

The interaction between sowing dates and organic extracts on yield and ear quality were significantly affected each other's. The highest values were recorded by sowing on 15 April with using 50 % compost + extracts of vermicompost or chicken manure. The lowest values were recorded with sowing on 5 June with adding 100 % compost.

Table 7: Effect of sowing dates and organic extracts on yield component of sweet corn plants during 2017 and 2018 seasons

Fertilizer treatments	Yield/plot (kg 6 m^{-2})							
	First season				Second season			
	Sowing dates							
	15-Apr	10-May	5-Jun	Mean	15-Apr	10-May	5-Jun	Mean
100 % C	12.85 e	13.06 e	10.94 f	12.29 D	14.50 f	12.93 g	11.29 h	12.91 D
50 % C + CE	16.51 d	16.11 d	12.61 e	15.08 C	19.20 c	15.93 e	13.00 g	16.04 C
50 % C + VE	20.75 a	19.65 b	16.55 d	18.98 A	22.31 a	19.46 c	17.06 de	19.61 A
50 % C + ChE	19.67 ab	18.15 c	15.52 d	17.78 B	20.70 b	17.97 d	16.00 e	18.22 B
Mean	17.44 A	16.74 B	13.90 C		19.18 A	16.57 B	14.34 C	
	Yield/plant (g)							
100 % C	450.00 f	451.67 f	405.33 g	435.67 D	483.33 f	434.00 g	376.00 h	431.11 D
50 % C + CE	578.00 de	557.33 e	467.00 f	534.11 C	640.00 c	520.00 ef	433.33 g	531.11 C
50 % C + VE	726.33 a	679.67 b	613.00 cd	673.00 A	743.67 a	633.67 c	568.67 d	648.67 A
50 % C + ChE	689.00 ab	627.67 c	574.67 de	630.45 B	690.00 b	573.33 d	533.33 de	598.89 B
Mean	610.83 A	579.09 B	515.00 C		639.3 A	540.3 B	477.8 C	

Means followed in same column by similar letters are not statistically different at 0.05 level according to Tukey test.
C = Compost; CE = Compost extract; VE = Vermicompost extract; ChE = Chicken manure extract

Table 8: Effect of sowing dates and organic extracts on the characteristics of sweet corn ears during 2017 and 2018 seasons

Fertilizer treatments	Ear diameter (cm)							
	First season				Second season			
	Sowing dates							
	15-Apr	10-May	5-Jun	Mean	15-Apr	10-May	5-Jun	Mean
100 % C	5.50 fg	5.27 g	4.93 h	5.23 D	5.50 g	5.50 g	5.03 h	5.34 D
50 % C + CE	6.00 bcd	5.70 ef	5.33 g	5.68 C	6.03 cd	5.97 cd	5.63 fg	5.88 C
50 % C + VE	6.37 a	6.17 b	5.93 cd	6.16 A	6.60 a	6.37 b	5.87 de	6.28 A
50 % C + ChE	6.27 ab	6.03 bc	5.73 def	6.01 B	6.40 ab	6.20 bc	5.73 ef	6.11 B
Mean	6.03 A	5.77 B	5.47 C		6.13 A	6.01 B	5.57 C	
	Ear length (cm)							
100 % C	24.33 fg	23.33 gh	21.33 h	23.00 C	25.67 gf	24.33 gh	22.33 h	24.11 D
50 % C + CE	28.67 de	26.67 ef	25.00 fg	26.78 C	29.33 de	27.33 ef	26.33 fg	27.67 C
50 % C + VE	35.33 a	33.00 ab	31.33 bcd	33.22 A	36.33 a	34.00 ab	32.33 bc	34.22 A
50 % C + ChE	33.33 ab	32.33 bc	30.00 cd	30.92 B	34.33 ab	33.00 bc	30.67 cd	32.67 B
Mean	30.42 A	28.83 B	26.92 C		31.42 A	29.67 B	27.92 C	
	Ear mass (g)							
100 % C	224.67 f	226.00 f	202.67 g	217.78 D	241.67 f	216.67 g	188.33 h	215.56 D
50 % C + CE	289.00 de	278.33 e	233.33 f	266.89 C	320.00 c	260.00 ef	216.67 g	265.56 C
50 % C + VE	363.00 a	339.67 b	306.67 cd	336.44 A	371.67 a	316.67 c	284.33 d	324.22 A
50 % C + ChE	344.00 ab	313.67 c	287.33 e	315.00 B	345.00 bcd	286.67 d	266.67 de	299.44 B
Mean	305.17 A	289.42 B	257.50 C		319.58 A	270.00 B	239.00 C	

Means followed in same column by similar letters are not statistically different at 0.05 level according to Tukey test.
C = Compost; CE = Compost extract; VE = Vermicompost extract; ChE = Chicken manure extract

3.4 KERNEL COMPOSITIONS

Data in Table 9 showed the effects of organic extract treatments on kernel compositions for three sowing dates of sweet corn in the two seasons. The highest percentage of TSS, total sugars and dry matter in kernels was obtained by sowing on 15 April. The plants that planted on 10 May came in second order, whereas the plants that planted on 5 June gave the lowest values. This result may be due to that the weather conditions in the early sowing were the most favorable to plant growth, which increasing the vegetative growth and photosynthesis products that translocation to corn kernels (Maddonni et al., 2004, Ibrahim et al., 2013, Maga et al., 2015).

All treatments of 50 % compost + any organic extracts increased kernel compositions of TSS, total sugars and dry matter compared to 100 % compost treatment. The highest values of kernel compositions was produced by 50 % compost + vermicompost extract. This result may be attributed to the positive role of the vermicompost extract in improving vegetable growth and nutritive status of plants as show in Tables 4, 5 and 6, which led to increase photosynthesis products that translocation to corn kernels. These results are harmony with those reported by Bulalin et al. (2015), Kovacik et al. (2015) and Rogelio (2017).

The interaction between sowing dates and organic extracts had significant effect on kernel compositions. The highest values of TSS, total sugars and dry matter were recorded with sowing on 15 April with using 50 % compost + vermicompost extract. The lowest values were recorded with sowing on 5 June with adding 100 % compost.

4 CONCLUSION

It could be concluded that early sowing on April had improved the growth, yield and quality of sweet corn compared to sowing during May and June. As well as, substituting the full dose of compost by half dose of compost with adding any extracts of compost, vermicompost

Table 9: Effect of sowing dates and organic extracts on the characteristics of sweet corn ears during 2017 and 2018 seasons

	TSS in kernels (%)							
	First season				Second season			
	Sowing dates							
Fertilizer treatments	15-Apr	10-May	5-Jun	Mean	15-Apr	10-May	5-Jun	Mean
100 % C	16.00 g	15.93 g	15.57 H	15.83 D	15.97 fg	15.90 fg	15.60 g	15.82 C
50 % C + CE	16.67 de	16.47 ef	16.20 fg	16.44 C	16.50 de	16.47 de	16.13 ef	16.37 B
50 % C + VE	17.57 a	17.47 ab	17.17 bc	17.40 A	17.53 a	17.37 ab	17.07 bc	17.32 A
50 % C + ChE	17.40 ab	17.13 bc	17.00 cd	17.18 B	17.37 ab	17.13 bc	16.83 cd	17.11 A
Mean	16.91 A	16.75 B	16.48 C		16.84 A	16.72 AB	16.41 C	
	Total sugars (%)							
100 % C	12.30 e	12.19 e	11.53 f	12.01 C	12.18 e	12.13 e	11.58 f	11.97 C
50 % C + CE	12.72 d	12.59 d	12.19 e	12.50 B	12.59 d	12.56 d	12.14 e	12.43 B
50 % C + VE	13.41 a	13.33 ab	12.99 c	13.24 A	13.38 a	13.13 ab	12.93 bc	13.15 A
50 % C + ChE	13.28 ab	13.08 bc	12.65 d	13.00 B	13.25 a	13.07 ab	12.73 cd	13.02 A
Mean	12.93 A	12.80 AB	12.34 B		12.85 A	12.73 AB	12.35 C	
	Dry matter in kernels (%)							
100 % C	22.37 e	22.33 e	22.02 e	22.24 D	22.27 e	22.15 ef	21.83 f	22.08 D
50 % C + CE	23.22 cd	23.08 d	23.05 d	23.12 C	23.12 cd	22.98 d	22.95 d	23.02 C
50 % C + VE	24.10 a	23.81 ab	23.65 b	23.86 A	24.00 a	23.69 ab	23.53 b	23.74 A
50 % C + ChE	23.69 ab	23.65 b	23.59 bc	23.64 B	23.59 ab	23.52 b	23.47 bc	23.53 B
Mean	23.35 A	23.22 B	23.08 C		23.25 A	23.09 B	22.95 C	

Means followed in same column by similar letters are not statistically different at 0.05 level according to Tukey test.
C = Compost; CE = Compost extract; VE = Vermicompost extract; ChE = Chicken manure extract

or chicken manure had improved yield and quality of sweet corn. The plants that were sown early (April 15) and were given half dose of compost + vermicompost extract produced the highest yield and quality of sweet corn ears.

5 ACKNOWLEDGEMENT

This work has been supported by Central Laboratory for Organic Agriculture, Central Laboratory for Agricultural Climate, Agricultural Research Center.

6 REFERENCES

Abbasi, P. A., Al-Dahmani, J., Sahin, F., Hoitink, H. A. J. & Miller, S. A. (2002). Effect of compost amendments on disease severity and yield of tomato in conventional and organic production systems. *Plant Disease*, *86*, 156-161. https://doi.org/10.1094/PDIS.2002.86.2.156

Abou-El-Hassan S., Abdrabbo, M. A. A. & Desoky, A. H. (2014). Enhancing Organic Production of Cucumber by using Plant Growth Promoting Rizobacteria and Compost Tea under Sandy Soil Condition. *Research Journal of Agriculture and Biological Sciences*, *10*, 162-169.

Abou-El-Hassan, S., El-Behairy, U. A., El-Shinawy, M. Z. & Abou-Hadid A. F. (2002). Effect of using organic manure compostea as a nutrient solution on quality of cantaloupe fruits in nutrient film technique (NFT). *Egyptian Journal of Horticulture*, *29*, 461-472.

Abou-El-Hassan, S. (2010). *Studies on using different compost teas as nutrient solutions for cantaloupe production with nutrient film technique*. Ph.D. Thesis Faculty of Agriculture. Ain Shams University, Egypt.

Anapalli, S. S., Ma, L., Nielsen, D. C., Vigil, M. F. & Ahuja, L. R. (2005). Simulating planting date effects on corn production using RZWQM and CERES-Maize models. *Agronomy Journal*, *97*, 58-71. https://doi.org/10.2134/agronj2005.0058

Arancon, N. Q., Edward, C. A., Dick, R. & Dick, L. (2007). Vermicompost tea production and plant growth impacts. *BioCycle*, 48, 51-52.

AOAC (2005). *Official Methods of Analysis*, 18thed., Association of Official Agricultural Chemists.12thed., Gaithersburg, D.C., USA.

Biocycle (2004). Building a knowledge base for compost tea. *Journal of Composting and Organic Recycling*, June, 1-2.

Bulalin, S. P., Llamelo, N., Pattung, A., Ocampo, R., Bangyad, S., Digma, G. & Corpuz, P. (2015). Enhancing corn productivity through application of vermi tea as foliar spray. *Asia Pacific Journal of Multidisciplinary Research*, *3*, 74-81.

Cottenie, A., Verloo, M., Kiekers, L., Velghe, G. & Camrbynek, R. (1982). *Chemical Analysis of Plants and Soils*. Hand Book, 1-63, Ghent, Belgium.

Edwards, C. A., Arancon, N. Q. & Greytak, S. (2006). Effects of vermicompost teas on plant growth and disease. *BioCycle*, *47*, 28-31.

Edwards, C. A. & Neuhauser, E. F. (1988). *Earthworm in waste and environmental management*. ISPB Academic Publication. The Hague, Netherlands PP211-220.

El-Tantawy, I. M., El-Ghamry, A. M. & Habib, A. H. (2009). Effect of chicken manure and manure compost tea on potato yield and soil fertility. *Journal of Agriculture Science*, *Mansoura University*, *34*, 659-668. https://doi.org/10.21608/jssae.2009.90265

El-Shinawy, M. Z., Abd-Elmoniem, E. M. & Abou-Hadid, A. F. (1999). The use of Organic manure for lettuce plants grown under NFT conditions. *Acta Horticulturae*, *491*, 315-318. https://doi.org/10.17660/ActaHortic.1999.491.47

FAO (1980). *Soil and Plant Analysis*. Soils Bulletin 38/2,250P.

FiBL & IFOAM (2018). *The World of Organic Agriculture*, Statistics and Emerging Trends.

Gharib, F. A., Moussa, L. A. & Massoud, O. (2008). Effect of compost and bio-fertilizers on growth, yield and essential oil of sweet Marjoram (*Majorana hortensis*) plant. *International Journal of Agriculture and Biology*, *10*, 381-387.

Ghobrial, W. N., Mehesen, A. A., Abass, J. M., Shalaby, M. E. & Omar, A. F. (2009). Potential impacts of rhizobium and compost tea enriched with rhizobacteria for enhancing production of faba bean against Broad bean mottle virus (BBMV). *Journal of Agriculture Research. Kafer El-Sheikh University*, *35*, 20-38.

Gross, A., Arusi, R., Fine, P. & Nejidat, A. (2008). Assessment of extraction methods with fowl manure for the production of liquid organic fertilizers. *Bioresource. Technology*, *99*, 327-334. https://doi.org/10.1016/j.biortech.2006.12.016

Ibrahim, H. A. K. & Ibrahim, S. M. (2014). Effect of Some organic extracts on essential nutrients uptake of sugar beet under saline conditions. *Research Journal of Agriculture and Biological Sciences*, *10*, 53-64.

Ibrahim, H. A., El-Behairy, U. A., El-Sawi Lashine, Z. A., EL-Desuki, M. Bakry M. O. & El-Shorbagy, T. (2013). Effect of sowing date and potassiumf application on growth, yield and quality of sweet corn (*Zea mays* var. *rugosa*). *Journal of Applied Sciences Research*, *9*, 5126-5135.

Insam, H. & de Bertoldi, M. (2007). Microbiology of the composting process. In L.F. Diaz, M. de Bertoldi, W. Bidlingmaier and E. Stentiford (Eds.), *Waste Management Series*, *8*, 25-48. Amsterdam, The Netherlands: Elsevier. https://doi.org/10.1016/S1478-7482(07)80006-6

Kamara ,Y., Ekeleme, F., Chikoye, D. & Omoigui, L. O. (2009). Planting date and cultivar effects on grain yield in dryland corn production. *Agronomy Journal*, *101*, 91-98. https://doi.org/10.2134/agronj2008.0090

Keeling, A. A., Mc-Callum, K. R. & Beckwith, C. P. (2003). Mature green waste compost enhances growth and nitrogen uptake in wheat and oilseed rape through the action of water extractable factors. *Bioresource Technology*, *90*, 127-132. https://doi.org/10.1016/S0960-8524(03)00125-1

Kovacik, P., Renco, M., Slmansky, V., Hanackova, E. & Wisniowska-Kielian, B. (2015). Impacts of vermicompost extract application into soil and on plant leaves on maize phytomass formation. *Journal of Ecological Engineering*, *16*, 143-153. https://doi.org/10.12911/22998993/59363

Kaur, N. & Kaur, P. (2018). Sowing date effects on growth characteristics of two maize cultivars. *Agricultural Research Journal*, *55*, 251-257. https://doi.org/10.5958/2395-146X.2018.00047.9

Lopez-Espinosa, S. T., Moreno-Resendez, A., Cano-Ríos, P., Rodriguez-Dimas, N., Robledo-Torres, V. & Marquez-Quiroz, C. (2013). Organic fertilization: An alternative to produce jalapen pepper under greenhouse conditions. *Emirates Journal of Food and Agriculture*, *25*, 666-672. https://doi.org/10.9755/ejfa.v25i9.15979

Maddonni, G. A., Otegui, M. E. & Bonhomme, R. (2004). Grain yield components in maize: II. Postsilking growth and kernel weight. *Field Crops Research*, *56*, 257-264. https://doi.org/10.1016/S0378-4290(97)00094-4

Mahmoud, M. M., (2011). Manure tea or amino acids as potential economic and safe substitutes for mineral and organic fertilization and the effect on growth, yield and chemical composition of faba bean plants. *Journal of Soil Science and Agricultural Engineering, Mansoura University*, *2*, 1059-1073. https://doi.org/10.21608/jssae.2011.56054

Maga, T. J., Vange, T. & Ogwuche, J. O. (2015). The Influence of Sowing Dates on the Growth and Yield of Two Maize (*Zea mays* L.) Varieties Cultivated under Southern Guinea Savannah Agro-Ecological Zone. *American Journal of Experimental Agriculture*, *5*, 200-208. https://doi.org/10.9734/AJEA/2015/12661

Martens, D. A., (2000). Management and crop residue influence soil aggregate stability. *Journal of Environmental Quality*,, *29*, 723–727. https://doi.org/10.2134/jeq2000.00472425002900030006x

Meshref, H. A., Rabie, M. H., El-Ghamry, A. M. & El-Agamy, M. A. (2010). Maximizing utilization of compost addition using foliar compost extract and humic substances in alluvial soil. *Journal of Soil Science and Agricultural Engineering, Mansoura University*, *1*, 957-971. https://doi.org/10.21608/jssae.2010.75464

Namakka, A., Abubakar, I. U., Sadik, A. I., Sharifai, A. I. & Hassas, A. H. (2008). Effect of sowing date and Nitrogen level on yield and yield components of two extra early maize varieties (*Zea mays* L.) in Sudan Savanna of Nigeria. *ARPN Journal of Agricultural and Biological Science*, *3*, 1-5.

Nawar, D. A. S. & Ibraheim, S. Kh. A. (2014). Effect of algae extract and nitrogen fertilizer rates on growth and productivity of peas. *Middle East Journal of Agriculture Research*, *3*, 1232-1241.

Nielson, R. L., Thomison, P. R., Brown, G. A., Halter, A. L., Wells, J. & Wuethrich, K. L. (2002). Delayed planting date effects on flowering and grain maturation of corn. *Agronomy Journal*, *94*, 549-558. https://doi.org/10.2134/agronj2002.5490

Pant, A., Radovich, T. J. K., Hue, N. V. & Arancon, N. Q. (2011). Effects of vermicompost tea (aqueous extract) on pak choi yield, quality and on soil biological properties. *Compost Science & Utilization*, *19*, 279-292. https://doi.org/10.1080/1065657X.2011.10737010

Peykarestan, B. & Seify, M. (2012). Sowing date effect on growth and yield attributes of corn (*Zea mays everta* Sturt.) grown under different densities. *African Journal of Agricultural Research*, *7*, 4427-4431. https://doi.org/10.5897/AJAR12.978

Pokhrel, B., Sorensen, J. N. & Petersen, K. K. (2017). Effect of plant based organic fertilizers and chicken manure extract on plant growth and root zone activities of tomato. *Acta Horticulturae*, *1164*, 173-180. https://doi.org/10.17660/ActaHortic.2017.1164.22

Rah Khosravani, A. T., Mansourifar, C., Modarres Sanavy, S. A. M., Asilan, K. S. & Keshavarz, H. (2017). Effects of sowing date on physiological characteristics, yield and yield components for different maize (*Zea mays* L.) hybrids. *Notulae Scientia Biologicae*, *9*,143-147. https://doi.org/10.15835/nsb919913

ROU, (2007). Recycled Organics Unit, Overview of compost tea use in New South Wales. 2nd ed. http://www.recycledorganics.com

Rogelio, R. M., (2017). Alternative growth enhancers for corn production: Efficacy of vermitea on sweet corn (*Z. mays* var. *saccharata* Sturt.) production. *International Journal of Advanced Engineering and Management Research*, *2*, 552-567.

Seran, T. H. & Shahardeen, R. N. M. (2013). Marketable pod yield of vegetable cowpea (*Vigna unguiculata*) as influenced by organic manures fermented with EM solution. *The Open Horticulture Journal*, *6*, 19-23.

Shaheen, A. M., Rizk, Fatma A., Abd El-Samad, E. H., Mahmoud, S. H. & Salama, Dina M. (2018). Chicken manure tea and effective micro-organisms enhanced growth and productivity of common bean plants. *Middle East Journal of Agriculture Research*, *7*, 1419-1430.

Snedecor, G. W. & Cochran, W. G. (1980). *Statistical methods*. Sixth Edition, Iowa state university press, Ames., Iowa, U.S.A.

Thakur, G. D., Bavalgave, V. G., Waghmare, M. S., Kesare, B. J. & Khandekar, B. S. (2009). Effect of fertilizer levels on growth and yield of sweet corn. *International Journal of Agriculture Sciences*, *5*, 100-102.

doi:10.14720/aas.2020.116.1.1181 Original research article / izvirni znanstveni članek

Genetic studies of soybean [*Glycine max* (L.) Merr.] response to seed storage stress factors

Gbemisola OKUNLOLA [1, 2], Emmanuel IDEHEN [1], Johnson ADETUMBI [3], Christopher ALAKE [1], Solomon AKINYOSOYE [3], Oluwafemi AMUSA [4]

Received June 03, 2019; accepted June 03, 2020.
Delo je prispelo 03. junij 2019; sprejeto 03. junij 2020

Genetic studies of soybean [*Glycine max* (L.) Merr.] response to seed storage stress factors

Abstract: Soybean [*Glycine max* (L.) Merr.] responded differently to storage stress factors. This research work aimed at assessing genetic potentials of fifteen soybean varieties to storage stress via accelerated aging technique and grouping them based on their levels of tolerance to storage stress using simple sequence repeat (SSR). Tolerance of the seed of 15 soybean varieties to storage stress was assessed by subjecting them to accelerated aging for 0, 6, 12, and 24 hours at 42 °C temperature and 100 % relative humidity (RH), after which their quality was assessed to determine their tolerance to storage stress. Same varieties were also stored for 6 months under ambient environment at 65 ± 5 % R.H and 25 ± 2 °C and they were genotyped with 19 simple sequence repeat (SSR) markers. The varieties were grouped on the basis of their levels of tolerance to storage stress. The principal components analysis (PCA) showed that germination rate index (GRI) and germination index (GI) were the major indices responsible for the significant variation in the seedling vigor characters. TGX1835-10E and TGX1448-2E were identified as varieties with good storage ability and therefore recommended for storage improvement in soybean breeding programs. Six SSR markers (Satt 565, Satt 175, Satt 281, Satt 600, Satt 160 and Satt 281) were identified as candidate markers for detection of alleles for tolerance to storage stress.

Key words: soybean; seed accelerated aging; principal components analysis (PCA); dendrogram; SSR markers; tolerance

Genetske raziskave soje [*Glycine max* (L.) Merr.] v povezavi s stresnimi dejavniki pri shranjevanju semena

Izvleček: Genotipi soje (*Glycine max* (L.) Merill) se odzivajo različno na stresne dejavnike med shranjevanjem semena. Namen raziskave je bil oceniti genetski potencial 15 sort semen soje na stres med shranjevanjem pri pospešenih tehnikah staranja. Genotipi so bili razvrščeni na osnovi njihove tolerance na stres med staranjem na osnovi enostavnih ponavljajočih se zaporedij (SSR) in agronomskih lastnosti. Toleranca 15 sort semen soje na stres med shranjevanjem je bila ocenjena z izpostavitvijo pospešenemu staranju za 0, 6, 12, in 24 ur pri 42 °C in 100 % relativni vlažnosti (RH). Nekatere od sort so bile shranjene tudi za 6 mesecev v razmerah 65 ± 5 % relativne vlažnosti in pri 25 ± 2 °C. Ti genotipi so bili ovrednoteni z markerji na osnovi19 enostavnih ponavljajočih se zaporedij (SSR). Sorte so se združevale na osnovi njihove tolerance na stres med shranjevanjem. Analiza glavnih komponent je pokazala (PCA), da sta bila indeks hitrosti kalitve (GRI) in kalitveni indeks (GI) najpomembnejša pokazatelja značilne variabilnosti v lastnostih, ki označujejo vigor sejank. TGX1835-10E in TGX1448-2E sta bili prepoznani kot sorti z dobro sposobnostjo shranjevanja in sta priporočeni v žlahtniteljskih programih soje za izboljševanje shranjevanja semena. Šest SSR markerjev (Satt 565, Satt 175, Satt 281, Satt 600, Satt 160 and Satt 281)je bilo prepoznanih kot primernih za ugotavljanje alelov odgovornih za toleranco na stres med shranjevanjem.

Ključne besede: soja; pospešeno staranje semen; analiza glavnih komponent (PCA); dendrogram; SSR markerji; toleranca

---

[1] Federal University of Agriculture, Crop Improvement and Seed Enterprise Development, Abeokuta, Nigeria
[2] Corresponding author, e-mail: gbemisolaokunlola1@yahoo.com
[3] Obafemi Awolowo University, Institute of Agricultural Research and Training, Ibadan, Nigeria
[4] University of Lagos, Department of Cell Biology and Genetics, Nigeria

1 INTRODUCTION

Soybean *(Glycine max* (L.) Merrill) is one of the oldest cultivated leguminous crops (Verma and Shoemaker, 1996). It is one of the most important economical legumes in the world, providing protein and oil to food and animal feed industries, as well as base ingredients for hundreds of chemical products (Hedley, 2001). It is grown for its beans, which have numerous uses (Camara, 2000). The beans can be processed into various meals, such as soy meat, cakes, baby foods, "tofu," and "dawadawa"- a local seasoning product for stews and soups (Abbey et al., 2001). The ability of the crop to increase dietary quality of resource-poor people all over the world (Hartman et al; 2011) has increased the demand for the beans with equivalent increase in its production. Provision of sufficient high-quality seed to farmers at an affordable price at the right time is a big challenge because the seeds deteriorate rapidly in quality during storage and therefore soybean farmers face inadequate supply of quality seed during the planting season (Dugjeet al., 2009). However, it has been reported that loss of germination potential of soybean seed is more acute in tropical and sub-tropical regions of the world when compared with temperate environments (Bhatia, 1996). This is because temperature, which is one of the major storage stress factor affects the storability of the seeds and also causes chemical changes, such as hydrolytic and oxidative rancidity (changes in oil quality) in the seeds, leading to reduced seed quality and subsequent loss of viability across time (Baskin and Delouche, 1973). This resulted into farmers planting available seed that often has low vigor, which in turn affects the yield (Nkang and Umoh, 1997). It has been proved that cultivars respond differently to environment situations due to their genetic variability (Flajšman et al., 2019; Badu-Apraku et al., 2017). Hence researchers have explored the use genetic variability among germplasms to solve genetic and agronomic problems.

Principal component analysis (PCA) and cluster analysis are methods of statistical grouping of germplasm, which provide information regarding the contributions of each character to total variation and group individuals based on the their genetic attributes respectively. These two techniques aid effective selection by plant breeders and or geneticists in crop improvement programs. In solving problem of soybean seed deterioration during storage, there is need to understand genetic traits responsible for tolerance to storage stress, hence this research work aimed at assessing genetic constituents of fifteen soybean varieties and their response to storage stress via accelerated aging technique with a view to grouping them based on their levels of tolerance to storage stress using Simple Sequence Repeat (SSR) prim-

Table 1: List of Fifteen varieties of soybean and their attributes

Serial Number	Varietal Name	Source	Seed colour	Days to maturity	Initial Germination %	Maturity group
1	TGX1990-52F	IITA	Milky White	95 - 102	97	I
2	TGX1989-48FN	IITA	Milky White	95 - 110	81	II
3	TGX1989-49FN	IITA	Milky White	95 - 110	95	II
4	TGX1835-10E	IITA	Milky White	81- 95	97	00
5	TGX1990-46F	IITA	Milky White	95 - 102	83	I
6	TGX1990-95F	IITA	Milky White	95 - 110	84	II
7	TGX1987-19F	IITA	Milky White	95 -110	89	II
8	TGX1990-78F	IITA	Milky White	95 - 105	96	II
9	TGX1989-53FN	IITA	Milky White	95 - 105	95	II
10	TGX1989-75FN	IITA	Milky White	95 - 105	83	II
11	TGX1990-114FN	IITA	Milky White	108 - 115	88	II
12	TGX1990-110FN	IITA	Milky White	108 - 115	97	II
13	TGX1440-1E	IAR&T	Creamy	90-100	89	I
14	TGX1448-2E	IAR&T	Creamy	108-115	89	II
15	TGX1740-1E	IAR&T	Creamy	90-100	79	I

IITA: International Institute of Tropical Agriculture
IAR&T: Institute of agricultural Training and Research
Maturity group classification was according to Yuesheng et al., 2006.

ers and identifying markers associated with tolerance or susceptible alleles in these soybean genotypes.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL MATERIAL AND SOURCES

Fifteen (15) soybean varieties were collected from International Institute of Tropical Agriculture (IITA) and Institute of Agricultural Research and Training, Obafemi Awolowo University (I.A.R & T), both in Ibadan, Nigeria (Table 1).

2.2 EVALUATION OF SEEDLING VIGOR CHARACTERISTICS

These soybean varieties were subjected to various seed quality assessment to determine their initial quality attributes. The tests conducted are**:**

Germination test: Fifty seeds in three replicates of each variety were planted in seed bowls filled with adequately moistened river sand. Germinated seedlings were counted daily as from 3rd to 6th day after sowing. Percentage germination (G %) was determined by finding the ratio of normal germinated seed at 6 days after sowing to total number of seeds planted according to the method suggested by ISTA (1996) using Equation 1 :

$$\%G = \frac{\text{No of normal seedlings that germinated}}{\text{Total number of seeds planted}} \times 100$$

(Equation 1)

Seedling vigor assessment: Seedling vigor of the germinated seeds was assessed using the seedling vigor parameters suggested by various past researchers using equations (2) – (5)

Coefficient of velocity of Germination (*CVG*): This is an estimate of the rapidity of germination of the seed lot and it was estimated according to the method described by Scott et al. (1884).

$$CVG = \frac{\Sigma Ni}{\Sigma NiTi} x100$$

(Equation 2)

Where: N is the number of seeds germinated each day and T is the number of days corresponding to N

Germination Index (GI): This is an index of the speed of germination. This was calculated based on method described by Akande et al. (2012).

$$GI = \frac{\Sigma\,(\mathrm{Nx})(\mathrm{DAP})}{\text{Total number of normal seedlings that emerged on final day}}$$

(Equation 3)

where Nx is the number of normal seedlings that emerged on day x after seeding and DAP is days after planting

*Germination Rate Index (GRI)***:** This reflects the percentage of germination on each day of the germination period and was calculated according to the method of Olisa et al. (2010). Higher *GRI* values indicate higher and faster germination (Kedar, 2005).

$$GRI = \frac{G1}{x} + \frac{G2}{x} + \frac{G3}{x} + \cdots \frac{Gx}{x}$$

(Equation 4)

where, *G* = germination on each day after seed placement 1, 2, x = corresponding day of germination.

Seedling vigor Index (SVI): Seedling length was measured from five randomly selected seedlings of each replicate from the soil level at 3 and 6 days after planting. The SVI was then calculated based on method of Adebisi et al. (2004).

$$SVI = \frac{(Germination\ \%\ x\ Seedling\ length)}{100}$$

(Equation 5)

2.3 ACCELERATED AGING PROCEDURE, GERMINATION AND VIGOR TEST

Twenty five grams (25 g) of seeds in three replicates of the 15 soybean varieties were artificially aged using plastic boxes (11.0 × 11.0 × 3.5 cm) at 100 % relative humidity and 42 °C temperature for 6, 12 and 24 hours (Jagadishet al., 2013). Germination and seedling vigor attributes of the seed lots were determined after each aging period and compared to initial seed quality. The difference between the two quality factors determines their tolerance ability.

2.4 AMBIENT SEED STORAGE

Fifty grams (50 g) of clean seed of each variety was packaged into paper envelopes and stored under ambient environment with average relative humidity (RH) of 65 ± 5 % and temperature of 25 ± 2 °C in the laboratory of Grain Legumes Improvement Programme of IAR & T Ibadan for six months (Demir et al., 2008). Each variety was replicated three times and the envelopes were arranged in Completely Randomized Design (CRD). Samples of the stored seed were evaluated after six months of storage using procedure described above.

The germination percentage after accelerated aging and six months ambient storage was deducted from initial germination percentage to estimate the resistance ability of each variety, which resulted to the percentage germination loss (G loss).

2.5 DNA EXTRACTION AND MOLECULAR ANALYSIS FOR STRESS TOLERANCE TRIAL

2.5.1 DNA extraction

Five grams of leave samples were harvested from the plant of each variety sown in the green house at about two weeks after planting and stored in ice, kept at -20 °C and later transported to Plant Science Laboratory of National Institute of Science Laboratory Technology, (NISLT) Ibadan, Nigeria for DNA extraction. Genomic DNA was extracted using the ZR Plant/Seed DNA MiniPrep™ kit. DNA quantity and quality was measured using Nanodrop® (ND-1000 spectrophotometer) at A $260/_{280}$ absorbance. The ratio of A260/280 absorbance and values obtained ranged between 1.80 and 2.0, indicates good quality DNAs.

2.5.2 Polymerase Chain Reaction

The SSR amplification was carried out with 20 Simple Sequence Repeat (SSR) primers in 11 µl reaction mixture consisting of 2.0 µl of template DNA, 1.0 *µl each of forward and reverse primers, 1.0 µl dNTP mix, 6.0 µl* PCR assay buffer. The PCR reactions were performed in a thermo-cycler with initial denaturation at 94 *°C for five minutes followed by 35 cycles of denaturation at 94 °C for one minute, annealing for 30 seconds at 47 °C (56* °C for Satt 565 and extension for 30 s at 72 *°C with a final extension for seven minutes. The amplified products were separated on 6.0* % polyacrylamide gel. Gels were run for 3 h at 75 V in 1X TBE buffer. DNA fragments were visualized under UV light and photographed using gel documentation system. An identified band (allele) on gels was binary coded as 1 or 0 to indicate their presence or absence respectively in soybean for each SSR primer. The names and sequences of the SSR primers are presented in Table 2.

2.6 DATA ANALYSIS

Data obtained from accelerated aging and ambient stor-

Table 2: The sequence and annealing temperature of the soybean SSR primers

	Sequence		
Name of primer	Forward	Reverse	AT (°C)
Satt423	TTC GCT TGG GTT CAG TTA CTT	GTT GGG GAA TTA AAA AAA TG	47
Satt414	GCG TAT TCC TAG TCA CAT GCT ATT TCA	GCG TCA TAA TAA TGC CTA GAA CAT AAA	47
Satt434	GCG TTC CGA TAT ACT ATA TAA TCC TAA T	GCG GGG TTA GTC TTT TTA TTT AAC TTA A	47
Satt285	GCG ACA TAT TGC ATT AAA AAC ATA CTT	GCG GAC TAA TTC TAT TTT ACA CCA ACA AC	47
Satt154	AGA TAC TAA CAA GAG GCA TAA AAC T	AAA GAA ACG GAA CTA ATA CTA CAT T	47
Satt002	TGT GGG TAA AAT AGA TAA AAA T	TCA TTT TGA ATC GTT GAA	47
Satt160	TCC CAC ACA GTT TTC ATA TAA TAT A	CAT CAA AAG TTT ATA ACG TGT AGA T	47
Satt565	GCG CCC GGA ACT TGT AAT AAC CTA AT	GCG CTC TCT TAT GAT GTT CAT AAT AA	56
Satt281	AAG CTC CAC ATG CAG TTC AAA AC	TGC ATG GCA CGA GAA AGA AGT A	47
Satt233	AAG CAT ACT CGT CGT AAC	GCG GTG CAA AGA TAT TAG AAA	47
Satt600	GCG CAG GAA AAA AAA ACG CTT TTA TT	GCG CAA TCC ACT AGG TGT TAA T	47
Satt434	GCG TTC CGA TAT ACT ATA TAA TCC TAA T	GCG GGG TTA GTC TTT TTA TTT AAC TTA A	47
Satt285	GCG ACA TAT TGC ATT AAA AAC ATA CTT	GCG GAC TAA TTC TAT TTT ACA CCA ACA AC	47
Satt142	GGA CAA CAA CAG CGT TTT TAC	TTT GCC ACA AAG TTA ATT AAT GTC	47
Satt545	CAA TGC CAT TCC ATA TTT GTT	CAA TTG CCC TAG TTT TGA TAG	56
Satt389	GCG GCT GGT GTA TGG TGA AAT CA	GCG CCA AAA CCA AAA GTT ATA TC	47
Satt431	GCG TGG CAC CCT TGA TAA ATA A	GCG CAC GAA AGT TTT TCT GTA ACA	47
Satt354	GCG AAA ATG GAC ACC AAA AGT AGT TA	GCG ATG CAC ATC AAT TAG AAT ATA CAA	47
Satt175	GAC CTC GCT CTC TGT TTC TCA T	GGT GAC CAC CCC TAT TCC TTA T	47
Satt194	GGG CCC AAC TGA TAT TTA ATT GTA A	GCG CTT TGT GTT CCG ATT TTG AT	47

AT- Annealing temperature (°C).

age were subjected to analysis of variance (ANOVA) using SAS™ software package. Means were separated using Duncan Multiple Range Test (DMRT) at 5 % level of significance. Principal component analysis was conducted using standardized data obtained from the accelerated aging characters to determine factors contributing to the variance. Components with Eigen values > 1.0 were selected and factors with contributing characters values of > 6 were considered relevant for principal component (Matus et al., 1999). Varieties were then clustered into groups using hierarchical clustering based on squared Euclidean distance using PAST v2.17 software (Hammer et al., 2001). Pearson's coefficients of correlations between germination loss and seedling vigor characters were determined using STAR 2.0.1 software.Genetic diversity, Polymorphic information content (PIC), gene diversity, heterozygosity, percentage polymorphism, inbreeding coefficient and average number of alleles were estimated using Power Marker v3.0 software (Liu and Muse, 2005). Cluster analysis based on Euclidean distance coefficient was obtained with the unweighted pair-group method based on the arithmetic mean (UPGMA) to generate the dendrogram using PAST v2.17 software (Hammer et al., 2001).

3 RESULTS

3.1 RESPONSE OF SOYBEAN VARIETIES TO STORAGE STRESS

The mean square values for germination loss (G loss), coefficient of velocity of germination (CVG), growth rate index (GRI), germination index (GI) and seedling vigor index

Table 3: Mean square of soybean viability and other seedling vigor characteristics

SOV	df	Gloss (%)	GI	GRI	CVG	SVI
Rep	2	42.1	5.62	2.63	48.4	14.88
Variety (V)	14	1616.59**	5831.03**	662.92**	233.18**	219.15**
Aging Period (A)	4	28786.24**	50587.99**	4723.55**	633.39**	3302.44**
V x A	56	550.62**	1072.86**	114.07**	75.35**	47.64**
Error	148	60.91	96.76	10.54	36.95	11.1

*, **significant at p = 0.05 and p = 0.01, respectively
SOV: Sources of variation; df: Degree of freedom; G loss: Germination loss; GI: Growth index; GRI: Growth rate index; CVG: Coefficient of velocity of germination; SVI: Seedling vigor index

Table 4: Mean values of germination loss and seedling vigor parameters of fifteen soybean varieties

Variety	G. loss(%)	GI	GRI	CVG	SVI
TGX 1190-52F	32.00^{ab}	85.73^{fg}	25.43^{g}	37.32^{bcd}	15.08^{ef}
TGX 1989-48FN	15.20^{g}	87.67^{efg}	27.26^{efg}	39.65^{abcd}	16.07^{def}
TGX 1989-49FN	18.93^{efg}	103.13^{c}	31.48^{c}	41.27^{ab}	17.57^{bcde}
TGX 1835-10E	1.60^{h}	136.47^{a}	43.45^{a}	43.82^{a}	24.4^{a}
TGX 1990-46F	22.4^{efd}	82.07^{hg}	25.46^{g}	40.41^{abc}	14.99^{ef}
TGX 1990-95F	32.80^{ab}	63.73^{i}	18.26^{i}	30.65^{e}	10.29^{g}
TGX 1987-19F	29.60^{bc}	76.00^{h}	22.64^{h}	36.63^{bcd}	14.09^{f}
TGX 1990-78F	24.53^{cde}	94.87^{de}	28.92^{def}	35.96^{cd}	17.83^{bcd}
TGX 1989-53FN	26.67^{bcd}	88.53^{efg}	26.68^{fg}	39.38^{abcd}	14.95^{ef}
TGX 1989-75FN	37.60^{a}	53.07^{j}	14.84^{j}	29.21^{e}	8.51^{g}
TGX 1990-114FN	13.33^{g}	98.80^{cd}	29.31^{cde}	37.84^{bcd}	19.69^{bc}
TGX 1990-110FN	27.2^{bcd}	90.53^{ef}	25.46^{g}	35.08^{d}	14.03^{f}
TGX 1440-1E	16.53^{fg}	98.20^{cd}	29.92^{cd}	39.79^{abcd}	17.09^{cde}
TGX 1448-2E	4.80^{h}	114.80^{b}	35.08^{b}	40.24^{abc}	19.78^{b}
TGX 1740-1E	13.33^{g}	89.73^{efg}	27.88^{defg}	40.92^{abc}	16.14^{def}

Mean followed by the same alphabets are not significantly different at p = 0.05
G loss: Germination loss; GI: Growth index; GRI: Growth rate index; CVG: Coefficient of velocity of germination; SVI: Seedling vigor index

(SVI) of the fifteen soybean varieties as affected by accelerated aging and storage period are presented in Table 3. There were highly significant differences among soybean varieties in respect to their response to storage stress (temperature and pressure) as expressed by the germination loss and all the seedling vigor characteristics (Table 3).

There were significant differences among the soybean varieties with respect to their reactions to storage stress as measured by the germination loss and other seedling vigor characteristics at $p = 0.05$ level of significance (Table 4). Germination loss among the varieties ranged from 1.6 % to 37.6 % (as a measure of the difference in germination % between untreated seed and seed after 6 month of aging period). High germination loss (37.60 %, 32 %, 32.8 %) was recorded in 'TGX1989-75FN', 'TGX1190-52F' and 'TGX1190-95F' respectively while low germination loss was observed in 'TGX1835-10E' (1.60) and 'TGX1448-2E' (4.80). There was no significant difference in the germination loss of 'TGX1989-48FN' (15.20), 'TGX 1989-49FN' (18.93), 'TGX1990-114FN' (13.33), 'TGX1440-1E-1E' (16.53) and 'TGX1740-1F' (13.33) (Table 4). Germination loss (G loss) decreased with increasing aging period, for instance the seeds had lost germination ability of about 63.73 % as at 6 months of aging period. Other seedling vigor parameters (GI, GRI, CVG and SVI) decreased with increasing aging period (6 hours to 6 months) (Table 5).

3.2 CLASSIFICATION OF SOYBEAN VARIETIES BASED ON RESPONSE OF THE SEEDS TO STORAGE STRESS

The principal component analysis (PCA) based on response of the soybean varieties to storage stress revealed four component axes with Eigen values that were greater than 1.0. These accounted for 93.97 % of the total variation. PC1 accounted for 76.16 % of the variation with germination rate index and germination index being the major factor while the second principal component (PC2) was responsible for about 17.81 % of the variation and was associated majorly with coefficient of velocity of germination and germination loss (Table 6). Soybean varieties were clustered into two major groups (A and B) based on hierarchical clustering using squared Euclidean distance. Cluster 1 contained two soybean varieties ((TGX1448-2E and TGX1835-10E). Cluster 2 was

Table 5: Effect of accelerated aging period on soybean seed viability

Aging period	G loss %	GI	GRI	CVG	SVI
0 hour	0.00^{d}	111.11^{ab}	32.23^{b}	33.98^{c}	17.97^{b}
6 hours	8.00^{c}	113.42^{a}	36.54^{a}	44.14^{a}	27.63^{a}
12 hours	10.22^{c}	108.27^{b}	32.47^{b}	37.08^{a}	12.22^{b}
24 hours	23.56^{b}	87.93^{c}	25.87^{c}	37.08^{b}	12.22^{c}
6 months	63.73^{a}	33.71^{d}	10.52^{d}	36.88^{b}	4.33^{d}

Mean followed by the same alphabets are not significantly different at p = 0.05
G loss: Germination loss; CVG: Coefficient of velocity of germination; GRI: Growth rate index;
GI: Growth index; SVI: Seedling vigor index

Table 6: Characters with respect to its principal component (PC), Eigen values and variation based on response of the seed to storage stress

Characters	PC 1	PC 2
Germination loss	-0.47	0.27
Germination index	0.50*	-0.13
Germination rate index	0.51*	-0.022
Coefficient of velocity of germination	0.22	0.96*
Seedling vigor index	0.47	-0.01
Eigen value	3.81	0.89
% Variance	76.16	17.81
Cumulative	76.16	93.97

*component contributors

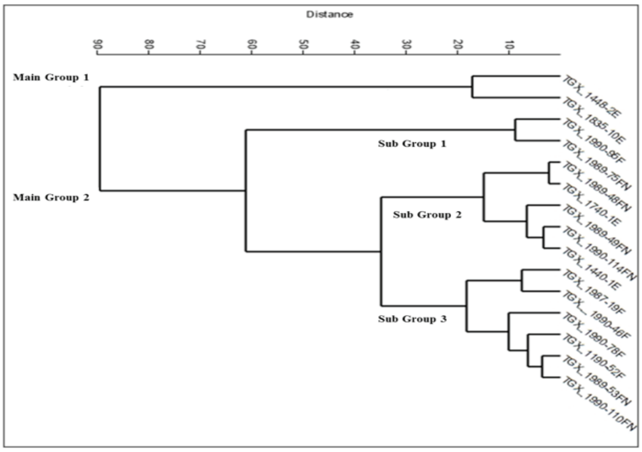


Figure 1: Dendrogram cluster grouping of 15 soybean varieties based on the response of the seed to storage stress

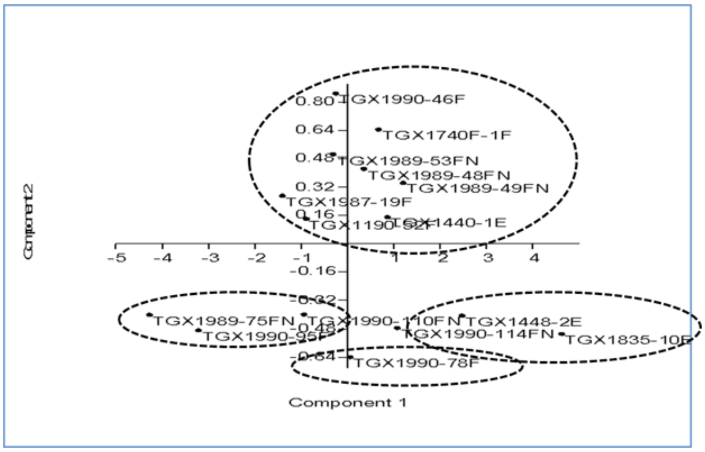


Figure 2: PCA scatter plot of soybean varieties evaluated based on the response of the seeds to storage stress on PC1 and PC2 axes

subdivided into three sub-groups. Sub-group 1 contained two varieties (TGX1990-95F and TGX1989-75EN), while sub-group 2 consisted of five varieties (TGX1989-49FN, TGX1740-1E, TGX1989-49FN, TGX1990-114FN and TGX1440-1E-1E) and sub-group 3 comprised six varieties (TGX1987-19F, TGX1990-46F, TGX1990-78F, TGX1190-52F, TGX1989-53FN and TGX1990-110FN) (Figure 1). The scatter plot of how the varieties are close to each other based on their response to storage stress shows that most of the varieties are similar to each other due to high germination loss after storage stress, hence they lied on the positive region of component 2. 'TGX 1448-2E', 'TGX 1990-114FN' and 'TGX 1835-10E' were similar to each other based on component 1, which is dominated by high germination indices after storage stress. 'TGX-1990-78F' was partially separated from other varieties along the lower negative region component 2 (Figure2).

3.3 CORRELATION BETWEEN PAIRS OF GERMINATION LOSS AND SEEDLING VIGOR PARAMETERS AT EACH AGING PERIOD

Correlation between pairs germination loss and seedling vigor parameters at each and across aging period showed that negative and significant association existed between pair of germination loss and seedling vigor parameters at 6 hours, 12 hours, 24 hours, 6 months and across aging period (-0.96** to -0.52*) (Table 7) Germination index (GI) correlated positively and significantly with germination growth index (GRI), coefficient of velocity of germination (CVG) and seedling vigor index (SVI), mostly at 12 hours, 24 hours, 6 months and across aging period (0.61* to 0.99**). Also, positive and significant relationship was recorded between pair of GRI with CVG and SVI, especially at 6 hours, 12 hours, 24 hours, 6 months and across aging period (0.50* to 0.99**). Similarly, CVG correlated significantly and positively with

Table 7: Pearson correlation between pairs of aging period and germination loss and seedling vigour parameters

	G loss %	GI	GRI	CVG	SVI
			Before		
Gloss	-	-	-	-	-
GI		-	0.93**	0.43	0.39
GRI			-	0.72**	0.29
CVG				-	-0.06
SVI					-
		6 hours			
	G loss	GI	GRI	CVG	SVI
Gloss	-	-0.52*	-0.88**	-0.79**	-0.86**
GI		-	0.47	0.61*	0.38
GRI			-	0.81**	0.96**
CVG				-	0.73**
SVI					-
		12 hours			
Gloss	-	-0.86**	-0.85**	-0.65**	-0.81**
GI		-	0.98**	0.72**	0.92**
GRI			-	0.79**	0.90**
CVG				-	0.61*
SVI					-
		24 hours			
Gloss	-	-0.96**	-0.95**	-0.68**	-0.92**
GI		-	0.99**	0.72**	0.96**
GRI			-	0.72**	0.96**
CVG				-	0.68*
SVI					-
		6 months			
	G loss	GI	GRI	CVG	SVI
Gloss	-	-0.85**	-0.96**	-0.42	-0.96**
GI		-	0.93**	0.51*	0.94**
GRI			-	0.50*	0.99**
CVG				-	0.52*
SVI					-
			Across		
Gloss	-	-0.80**	-0.89**	-0.88**	-0.89**
GI		-	0.85**	0.81**	0.82**
GRI			-	0.99**	0.97**
CVG				-	0.97**
SVI					-

* , **significant at $p = 0.05$ and $p = 0.01$, respectively
G loss: Germination loss; GI: Growth index; GRI: Growth rate index; CVG: Coefficient of velocity of germination; SVI: Seedling vigor index

SVI at 6 hours, 12 hours, 24 hours, 6 months and across aging period (0.52* to 0.97**).

3.4 CANDIDATE SSR MARKERS FOR DETECTION OF STORAGE STRESS TOLERANCE ALLELES IN SOYBEAN

3.4.1 Diversity studies

Eighteen out of the nineteen SSR primers (94.74 %) used in this study were polymorphic and were able to detect SSR markers linked with alleles associated with storage stress tolerance among soybean varieties. The polymorphic markers produced a total of 72 alleles in the 15 soybean varieties. The alleles ranged from two to seven per locus, with an average of four alleles per primer. Primers (Satt 389 and Satt 600) had the highest number of alleles (seven), and the rests had two to five alleles. Principal information content (PIC) values for the SSR markers ranged from 0.28 (SATT 160) to 0.81 (SATT 389) with an average of 0.57 (Table 8).

Five SSR markers (Satt175, Satt 600, Satt 190, Satt 565 and Satt 160) clearly discriminated the soybean varieties into tolerant and susceptible varieties. Satt 600 had two alleles (Satt 600_{100} and Satt 600_{140}) that linked with alleles in one of the tolerant variety (TGX1835-10E). Also, some other SSR primers like Satt 285 (Satt 285_{160} and Satt 285_{200}) were associated with alleles responsible for susceptibility to storage stress.

3.4.2 Genetic diversity of soybean varieties based on SSR molecular markers

Genetic distance among the 15 soybean varieties ranged from 0.069 to 0.514 in (TGX1989-75FN, and TGX1990-114FN) to (TGX1989-49FN and TGX1440-1E) respectively with a mean value of 0.265 (Table 9). The dendrogram grouped the soybean varieties into two main groups (A and B). The first main cluster (A) comprised 3 varieties (TGX1440-1E, TGX1448 and TGX1740) while the second main cluster (B) comprised twelve varieties, which was sub-divided into four sub-groups sub-cluster 2 had only one variety (TGX 1835-10E). Sub-cluster 3 comprised four varieties (TGX1190-52F, TGX1989-48FN, TGX1989-49FN and TGX1990-46F). Sub-cluster 4 had three varieties (TGX1990-46F, TGX1987-19F, TGX1990-78F) and sub-cluster 5 consisted of four vari-

Table 8: Major alleles and polymorphism information content of eighteen SSR markers tested on fifteen soybean varieties

S/No	Primer	MAF	Na	Na_{ave}	GD	He	MP	PP	%P	PIC	I
1	SATT423	0.40	4.00	2.07	0.66	0.80	0	4	100.00	0.59	-0.18
2	SATT414	0.50	3.00	2.40	0.62	1.00	2	1	33.33	0.55	-0.59
3	SATT434	0.54	3.00	2.47	0.57	0.93	0	3	100.00	0.49	-0.59
4	SATT285	0.50	3.00	3.00	0.50	1.00	3	0	0.00	0.38	-1.00
5	SATT154	0.38	3.00	1.47	0.66	0.69	0	3	100.00	0.59	-0.01
6	SATT002	0.50	3.00	2.20	0.58	1.00	2	1	33.33	0.49	-0.71
7	SATT160	0.83	4.00	1.07	0.30	0.25	0	4	100.00	0.28	0.20
8	SATT565	0.43	5.00	3.00	0.62	1.00	0	4	100.00	0.54	-0.60
9	SATT281	0.37	6.00	4.33	0.76	1.00	0	6	100.00	0.72	-0.29
10	SATT233	0.46	3.00	2.47	0.63	0.93	0	3	100.00	0.55	-0.46
11	SATT285	0.50	3.00	2.60	0.53	0.93	0	3	100.00	0.42	-0.72
12	SATT142	0.50	2.00	1.73	0.50	1.00	3	0	0.00	0.38	-1.00
13	SATT545	0.50	3.00	2.47	0.62	1.00	2	1	33.33	0.55	-0.58
14	SATT389	0.23	7.00	2.73	0.83	0.92	0	7	100.00	0.81	-0.07
15	SATT431	0.46	5.00	2.00	0.67	1.00	0	5	100.00	2.00	-0.46
16	SATT354	0.50	2.00	1.87	0.50	1.00	2	0	0.00	0.38	-1.00
17	SAAT175	0.40	6.00	2.07	0.69	1.00	0	6	100.00	0.64	-0.42
18	SATT600	0.33	7.00	1.80	0.76	0.67	0	7	100.00	0.73	0.16

MAF: major allele frequency; Na: number of observed alleles; Naave: average number of alleles; polymorphic alleles; % P: percentage polymorphism; PIC: polymorphic information content; I: inbreeding coefficient, He: heritability, MP: percentage monomorphism

Table 9: Distance indices among fifteen soybean varieties based on DNA analysis

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15
V1	-	0.111	0.153	0.375	0.292	0.194	0.236	0.181	0.167	0.139	0.181	0.333	0.306	0.222	0.292
V2		-	0.125	0.403	0.264	0.25	0.208	0.153	0.194	0.194	0.236	0.333	0.361	0.278	0.347
V3			-	0.361	0.278	0.236	0.194	0.167	0.125	0.208	0.222	0.236	0.347	0.292	0.278
V4				-	0.389	0.403	0.389	0.389	0.403	0.375	0.361	0.375	0.514	0.347	0.472
V5					-	0.292	0.194	0.222	0.319	0.236	0.25	0.347	0.264	0.292	0.333
V6						-	0.236	0.236	0.222	0.194	0.181	0.333	0.389	0.278	0.347
V7							-	0.167	0.208	0.181	0.194	0.236	0.236	0.264	0.25
V8								-	0.208	0.125	0.194	0.292	0.375	0.292	0.389
V9									-	0.111	0.125	0.167	0.361	0.25	0.264
V10										-	0.069	0.222	0.306	0.25	0.319
V11											-	0.181	0.292	0.264	0.306
V12												-	0.333	0.361	0.347
V13													-	0.306	0.208
V14														-	0.236
V15															-

V1: TGX1990-52F; V2: TGX1989-48FN; V3: TGX1989-49FN; V4: TGX1835-10E; V5: TGX1990-46F; V6: TGX1990-46F; V7: TGX1987-19F; V8: TGX1990-78F; V9: TGX1989-53FN; V10: TGX1989-75FN; V11: TGX1990-114FN; V12: TGX1990-110FN; V13: TGX1440-1E ; V14: TGX1448-2E; V15: TGX1740-1F

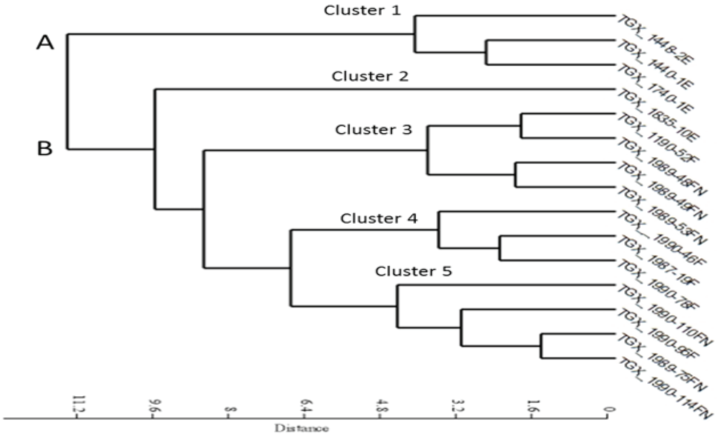


Figure 3: Dendrogram of 15 soybean varieties based on their genetic component identified using SSR markers

eties (TGX1990-110FN, TGX1990-95F, TGX1989-75FN, TGX1990-114FN) (Figure 3).

4 DISCUSSION

Seed storability is reported by Clerkx et al. (2004) to be a complex trait affected by environmental factors during seed formation, harvest and storage, and is usually controlled by several genes. The major storage stresses that seeds are exposed to are relative humidity and temperature. High germination loss and low seedling vigor parameters (GI, GRI, CVG and SVI) are indicators of low tolerance to storage stress imposed through accelerated aging and the ambient environment. Kehinde et al. (2013) found out that seed scientists have employed seeds viability and seedling vigor index to assess seed quality and the declined in these parameters have been associated with seed deterioration during storage. The significant variation observed in germination and other seedling vigor characteristics of the soybean varieties in this study can be attributed to the genetic makeup of the soybean varieties.

There were variations in the rate of deterioration of soybean varieties used in this study. This variation increased as the ageing duration increased. Similar result was reported by Jagadish et al. (2013) in the aging of forty soybean genotypes. This implies that seed deterioration of soybean is inevitable; however, varieties differed in their responses to aging factor that had been imposed on the seed due to storage stress. Also, it could be deduced that the significant variation observed in germination and other seedling vigor parameters under artificial aging treatments and ambient storage is due to different genetic makeup of the soybean varieties. Therefore, the diverse genetic constituent of the soybean varieties makes selection for storage stress tolerance possible. Adebisi et al. (2004) reported that seed quality and longevity performance are components of genetic make-up in soybean. Germination rate index and germination index accounted for the major factor to classify the varieties based on their tolerance to storage stress. This suggests that germination percentage is not the only factor that determines seed quality; therefore attention should be paid to these seedling vigor characters.

Simple correlation analysis has been considered adequate as a rough guide to the magnitude and direction of the relationships between two traits (Adebisi et al., 2010). High magnitude of coefficient of correlation (r) obtained among pairs of seedling vigor index at 6 hours, 12 hours, 24 hours, 6 months and across aging period, shows a strong association among the parameters and each parameter could be used to improve seedling vigor in cowpea.This corroborates with the findings of Brown and Caligari (2008) who reported that high and positive association between characters suggests that each of two parameters pairs could be controlled by closely linked genes, same or similar genes or by genes with pleiotropic effects on these parameters. However, negative and significant association was obtained between pair of germination loss and seedling vigor parameters at 6 hours, 12 hours, 24 hours, 6 months and across aging period. This implies that lower percentage germination loss leads to higher seedling growth parameters.

The clustering pattern of SSR markers agrees with the patterns obtained from accelerating aging imposed storage stress as regards grouping of susceptible soybean varieties to storage stress. Highly susceptible varieties (TGX 1990-95F and TGX 1989-75FN) were clustered together in all the trials, therefore this information confirms that these varieties will require improvement, if their cultivation will continue. SSR markers (Satt 600, Satt 285, Satt 175, Satt 190, Satt 565 and Satt 160) clearly distinguished the varieties into tolerant and susceptible varieties. These markers could have close linkage with good storability. SSR primers that were specifically associated with tolerance alleles (Satt 600) and susceptible alleles (Satt 285, Satt 281) identified were considered as good candidate markers for screening soybean germplasm for the identification of tolerance and susceptible genotypes in soybean breeding programmes. The results corroborates the work of Jagadish et al. (2013), Singh et al. (2008) and Dargahi et al. (2014) that identified same SSR markers to have linkage with storability traits in soybean. Hence, these markers are considered good candidate molecular markers for identifying alleles linked with tolerance or susceptibility to storage stress in soybean.

5 CONCLUSION

Soybean varieties differ in their response to storage stress. ‘TGX1835-10E’ and ‘TGX1448-2E’ were regarded as tolerant soybean varieties to storage stress as evident in the low germination loss recorded after subjecting them to accelerated ageing procedure. This is attributed to inherent genetic potentials to withstand storage stress. SSR markers Satt 565, Satt 175, Satt 281, Satt 600, Satt 160 and Satt 281 associated with storage stress in soybean seed. These candidate markers can be further studied on large soybean varieties to confirm their linkage with tolerant genes. This will be of immense uses to ascertaining their ability to soybean germplasm screening for seed storage tolerance breeding programs.

6 REFERENCES

Abbey, T. K. Essiah, J.W., Alhassan, A., Fometu, E., Ameyibor, K., and Wiredu, M. B. (2001). *Integrated Science for Senior Secondary School*. Unimax MacMillan Ltd. Ring Road South industrial Area Accra-North Ghana. Pp 177-178.

Adebisi, M. A., I. O. Daniel, I. O., M. O. Ajala, M. O. (2004a). Storage life of soybean seed after seed dressing. *Journal of Tropical Agriculture, 42*, 3-7.

Adebisi, M.A., Ajala, M.O., Ariyo. O.J. (2004b). Genetic studies on seed quality in sesame. *Proceedings of the 29th Annual Conference of the Genetics Society of Nigeria*. Pp. 209-212.

Adebisi, M.A., Okelola, F.S., Alake, C.O., Ayo-Vaughan, M.A., Ajala, M.O. (2010). Interrelationship between seed vigour traits and field performance in new rice for Africa (Nerica) genotypes (*Oryza sativa* L.). *Journal of Agricultural Science and Environment, 10*(2), 15-24.

Akande, S. R.,Olakojo, S. A., Ajayi, S. A., Owolade, O.F., Adetumbi, J. A., Adeniyan, O. N., Ogunbodede, B. A. (2012). Planting date effects on cowpea seed yield and quality at southern guinea Savanna of Nigeria. *Seed Tech*nology, *34*, 79-88.

Badu-Apraku, B., Yallou, C.G., Obeng-Antwi, K., Alidu, H., Talabi, A.O., Annor, B., Oyekunle, M., Akaogu. I.C. and Aderounmu, M. (2017).Yield gains in extra-early maize cultivars of three breeding eras under multiple environments. *Agronomy Journal, 109*(2), 418. https://doi.org/10.2134/agronj2016.10.0566

Baskin, C. C., Delouche, J.C. (1973). Accelerated aging techniques for predicting the relative storability of seed lots. *Seed Science & Technology, 14*, 427-452.

Bhatia V. S. (1996). Seed longevity as affected by field weathering and its association with seed coat and pod characters in soybean. *Seed Res*earch, *24*, 82-87.

Brown, J., and P. Caligari, (2008). *An introductionto plant breeding*. Blackwell Publishing Ltd, Oxford, UK. https://doi.org/10.1002/9781118685228

Camara, Y. (2000). *Profitability of cassava production system in West Africa: A comparative analysis* (Cote. D' Ivore, Ghana and Nigeria), Ph. D. Thesis, Michigan State University, East.

Clerkx, E.J.M., El-Lithy, M.E., Vierling, E., Ruys, G.J., Blankestijin-De Vries, H., Groot S.P.C., Vreugdenhil, D., Koornnee, F.M. (2004). Analysis of natural allelic variation of Arabidopsis seed germination and seedlongevity traits between the accessions Landsbergerecta and Shakdara, using a new recombinant inbred line population. *Plant Physiology*, *135,* 432–443. https://doi.org/10.1104/pp.103.036814

DargahiH.,Tanya, P., Srinives, P. (2014). Mapping of the genomic regions controlling seed storability in soybean (*Glycine max* (L.) Merill.). *Journal of Genet*ics, *93*, 365–370. https://doi.org/10.1007/s12041-014-0381-0

Demir, I., Mavi, K. (2008). Controlled deterioration and accelerated aging tests to estimate the relative storagepotential of Cucurbit seed lots. *Horticultural science*, *43*, 1544-1548. https://doi.org/10.21273/HORTSCI.43.5.1544

Dugje, I.Y., Omoigui, L.O., Ekeleme, F., Bandyopadhyay, R., Lava Kumar, P., Kamara, A.Y. (2009). *Farmers' Guide to Soybean Production in Northern Nigeria*. International Institute of Tropical Agriculture, Ibadan, Nigeria. 21 pp

Flajšman, M., Šantavec, I., Kolmanič, A., Košmelj, K., & Kocjan-Ačko, D. (2019). Agronomic performance and stability of seed, protein and oil yields of seven soybean cultivars determined in field experiments in Slovenia. *Genetika*, *51*(1), 31-46. https://doi.org/10.2298/GENSR1901031F

Hammer, D.A., Harper, T., Ryan, P.D. (2001). PAST: Palaeontological Statistics, version 2.17 software package for education and data analysis. *Palaeontologia electronica, 4*(1), 9 pp.

Hartman, G.L., West, E.D., Herman T. K. (2011). Crops that feed the World 2. Soybean worldwide production, use, and constraints caused by pathogens and pests. *Food Security, 3*, 5–17. https://doi.org/10.1007/s12571-010-0108-x

Hedley, C.L. ed. (2001*). Carbohydrates in Grain Legume Seeds: Improving Nutritional Quality and Agronomic Characteristics*. CABI Publishing: New York. https://doi.org/10.1079/9780851994673.0000

ISTA, (1996). *Handbook of Vigor Test Methods* (2ndEdn), International Seed Testing Association, Zurich,Switzerland, pp 28-37.

Jagadish, H.A, Kumar, M.B, Talukdar, A., Lal1, S.K., Dadlani, M. (2013). Molecular characterization and identification of candidate markers for seed longevity in soybean [*Glycine max* (L.) Merill]. *Indian Journal of Genetics,73*(1), 64-71. https://doi.org/10.5958/j.0019-5200.73.1.009

Kader, M.A. (2005). A comparison of seed germination calculation formulae and the associated interpretation of resulting data. *Journal and Proceedings of the Royal Society of New South Wale*, *138*, 65-75.

Kehinde, T.O., Ajala, M.O., Daniel, I.O., Oyelakin, O.O. (2013). Physiological and genetic integrity of amaranth (*Amaranthus* spp.) seed during storage. *International Journal of Plant Breeding and Seed Genetic, 7*(1), 35-46. https://doi.org/10.3923/ijpbg.2013.35.46

Liu, K. and Muse, S.V. (2005). PowerMaker: integrated analysis environment for genetic marker data. *Bioinformatics*, *21*(3), 2128-2129. https://doi.org/10.1093/bioinformatics/bti282

Matus, I., Gonzaléz, M.I., Pozo, A. (1999). An evaluation of phenotypic variation in a Chilean collection of garlic (*Allium sativum* L.) clones using multivariate analysis. *Plant Genetic Resources Newsletter, 117*, 31-36.

Nkang, A., Umho, E. O. (1996). Six month storability of five soybean cultivars as influenced by stage of harvest, storage temperature and relative humidity. *Seed Science and Technolology, 25*, 93-99.

Olisa, B.S., Ajayi, S.A., Akande, S.R. (2010). Physiological quality of seeds of promising African yam bean (Sphenomstylis stenocarpa (Hoechst. Ex A. Rich)Harms) and pigeon pea(*Cajanus cajan* (L.) Mill sp.) landraces. *Research Journal of Seed Sciences, 3*(2), 93-101. https://doi.org/10.3923/rjss.2010.93.101

Scott, S.J., Jones, R.A., Williams, W.A. (1984). Review of data analysis methods for seed germination. *Crop Science, 24*, 1192-l 199. https://doi.org/10.2135/cropsci1984.0011183X002400060043x

Singh, R. K., Raipuria, R. K., Bhatia, V. S., Rani, A., Pushpendra, T., Husain, S. M.,Satyavathi, C. T., Chauhan, G. S., Mohapatra, T. (2008). Identification of SSR markers associated with seed coat permeability and electrolyte leaching in soybean. *Physiology and Molecular Biology of Plants*, *14*, 173-177. https://doi.org/10.1007/s12298-008-0016-0

Statistical Tool for Agricultural Research (STAR, Version: 2.0.1, 2013 - 2020). Rice Research Institute (IRRI). http://bbi.irri.org

Verma, D.P.S., Shoemaker, R.C. eds. (1996). *Soybean Genetics, Molecular Biology and Biotechnology*. CAB International: Wallingford.

Yuesheng, W., Jianbing, Q., Junyi, G., Guangyuang, H. (2006). Classification and characteristic of maturity groups of Chinese landraces of Soybean [*Glycine max* (L.) Merr.]. Genetic Resources and Crop Evolution, *53*(4), 803–809. https://doi.org/10.1007/s10722-004-5731-y

doi:10.14720/aas.2020.116.1.1552

Genetic variability and selection criteria of some sunflower genotypes under different pollination methods

Mohamed Ali ABDELSATAR [1, 2], Asmaa Abd-EL-Halime AHMED [1], Suzan Abd El-Latif Kamel IBRAHIM [1]

Received March 5, 2020; accepted June 13, 2020.
Delo je prispelo 05. marec 2020, sprejeto 13. junij 2020

Genetic variability and selection criteria of some sunflower genotypes under different pollination methods

Abstract: To determinate self-compatible genotypes for increasing autogamy yielded, the ten sunflower genotypes were sown under four pollination methods i.e., open-, self-, hand- and sib-pollinations in a randomized complete block design replicated thrice at Kafr-El-Hamam Agricultural research Station, Sharkia Governorate, Agricultural Research Center, Egypt during 2018 and 2019 summer seasons. Significant variation existed for different pollination treatments, genotypes and their interactions for all studied traits. Among the treatments, open pollination followed by sibbing one was regarded as the best pollination treatment for head diameter, number of filled seeds per head, percentage of seed setting , kernel to hull ratio, seed mass per plant, seed yield per fed and seed oil content. Autogamy studies revealed that 'L120' followed by 'L92' and 'L880' recorded higher proportion of autogamy and self-compatibility. Preferred improvement of seed mass per plant may be achieved through selecting genotypes having the largest head diameter, bigger number of filled seeds per head, the highest proportion of seed setting and the heaviest seed, kernel and hull mass as proven by high phenotypic and genotypic coefficient of variation and high heritability coupled with high genetic advance (as % of mean) as well as correlation and path analyses at both phenotypic and genotypic levels.

Key words: autogamy; heritability; path analysis; sunflower

Genetska variabilnost in selekcijski kriteriji nekaterih genotipov sončnice pri različnih metodah opraševanja

Izvleček: Za določitev samokompatibilnih genotipov in povečanje samooploditve je bilo posejanih deset genotipov sončnice z različnimi načini opraševanja in sicer: navskrižno opraševanje, samoopraševanje, ročno opraševanje in opraševanje znotraj starševskih linij. Poskus je bil zasnovan kot naključni popolni bločni poskus s tremi ponovitvami na Kafr-El-Hamam Agricultural research Station, Sharkia Governorate, Agricultural Research Center, Egypt, v poletnih rastnih sezonah 2018 in 2019. Za vse preučevane lastnosti so se pokazale značilne razlike glede na način opraševanja, izbrane genotype in njihove interakcije. Med obravnavanji sta se navskrižno opraševanje in opraševanje znotraj starševskih linij izkazali kot najboljši način opraševanja glede na premer koška (glave) sončnic, število polnih zrn na košek, odstotek nastavka semen, razmerja med jedrci in luščinami, maso semen na rastlino, pridelek semena na fedan in vsebnost olja v semenu. Preučevanje avtogamije je odkrilo, da so imeli genotipi po vrstnem redu 'L120', 'L92' in 'L880' največji delež samooplodnje in samokompatibilnosti. Zaželjeno izboljšanje mase semena na rastlino bi lahko dosegli z izborom genotipov, ki imajo največji premer koška, večje število polnih semen na košek, največji nastavek semen, največjo maso semen, jedrc in luščin kot je bilo dokazano z velikim fenotipskim in genotipskim koeficientom variabilnosti in velike dednosti povezano z velikim napredkom v dedovanju (kot poprečni odstotek) kot tudi z analizami korelacije med preučevanimi lastnostmi na fenotipski in genotipski ravni.

Ključne besede: avtogamija; dednost; analiza povezanih znakov; sončnica

---

[1] Oil Crops Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt
[2] Corresponding author, e-mail: mohamedtemraz1@yahoo.com

1 INTRODUCTION

Sunflower is a major oilseed crop in the world after soybean and groundnut. Sunflower as cross-pollinated crops, which almost dependent on pollinating insects and wind for increasing seed yield. However, only pollinating insects and winds are not sufficient to cause maximum seed yield and quality. Thus, the major challenge of plant breeder is to determinate self-compatible genotypes for increasing autogamy or selfing yielded in the absence of pollinating insects. Self-compatible genotypes have higher yields than self-incompatible ones, when bees are not abundant. Consequently, cultivating self-compatible genotypes with high yielding ability offer a great opportunity to improve seed yield and oil quality of sunflower.

Pollination methods are important factor for identifying self-compatible genotypes with high yielding ability. In this concern, seed set under self-pollination conditions is strongly correlated with self-compatibility degree (Beg et al., 1987). Earlier findings of Sameena (2003) indicated that pollen blend treatment was the best one followed by open pollination, geitonogamy and autogamy, due to its enhanced seed setting rate, higher number of filled seeds per head, kernel to hull ratio and hence seed mass per plant as well as higher seed oil content. In the same context, maximum head diameter with less empty diameter, seed setting percentage, number of filled seed per head, 100 seed mass, seed yield per plant, per plot and per ha was obtained by combination of bee pollination and hand pollination methods than that of other pollination ones as recorded by Talele (2011) and Osman and Siham (2015).

Successful sunflower breeding program depends on magnitude of genetic variability in the breeding material and knowledge of selection criteria would be effective in improving seed yield. In this respect, considerable amount of genetic variability might be affect days to first flowering, days to full flowering, plant height, stem diameter, autogamy percentage, self-compatibility percentage, head diameter, number of filled seeds per head, number of unfilled seeds per head, total number of seeds per head, seed setting percentage, 100-seed mass, kernel mass (mg), hull mass (mg), hull content (%), kernel to hull ratio, seed mass per plant, seed yield per fed and seed oil content as reported by Sameena (2003), Supriya et al. (2016), Neelima et al. (2016) and Supriya et al. (2017). High heritability coupled with high percentage of genetic advance were observed for head diameter, seed mass per plant, 100-seed mass, pollen production per plant, plant height and percentage of hull mass as reported by by Khan and Muhammad (2007), Kalukhe et al. (2010), Sudrik et al. (2014) and Seyed et al. (2014), Sheshaiah and Shankargoud (2015), Supriya et al. (2016), Dudhe et al. (2017) and Baraiya et al. (2018).

Moreover, the selection criteria of correlation and path analyses at phenotypic and genotypic levels play a vital role in selected the best traits to improve seed mass per plant. Seed mass per plant highly significant and positively correlated with 100-seed mass, head diameter and number of filled seeds were reported by Sowmya et al. (2010), Neelima et al. (2012), Prabhakaran et al. (2013), Deengra et al. (2013) and Venkanna et al. (2014). Seed filling percentage, head diameter and hull content percentage exhibited good amount of direct effect on seed mass per plant and its correlation with these traits were also positively significant at both genotypic and phenotypic levels and it is indicated that these traits should be considered as selection criteria for higher seed yield as reported by Sowmya et al. (2010), Neelima et al. (2012), Tyagi and Khan (2013), Deengra et al. (2013) and Sanju et al. (2018).

Therefore, the present study aimed to find out the impact of pollination methods on seed yield and its attributes of some sunflower genotypes, to determinate self-compatible genotypes for increasing autogamy or selfing yielded in the absence of pollinating insects and to identify genetic variability among sunflower genotypes and selection criteria for improving yield.

2. MATERIALS AND METHODS

2.1 SITE DESCRIPTION

Four field experiments was conducted at the experimental Farm of Kafr-El-Hamam Research Station, Zagazig, Sharkia Governorate, Agricultural Research Center, Egypt ($30^{\backslash}$ 58^{0} N, $31^{\backslash}$ 50^{0}E) during the two successive summer seasons of 2018 and 2019.

2.2 EXPERIMENTAL DESIGN

The experiment was laid out in randomized complete block design for each pollination method with three replications. Pollination methods details were practiced as follow; open pollination (OP) was done by left head of plant for open pollination without bagging to allow self and cross-pollination, self-pollination (SP) was done by bagging sunflower heads before commencement of flowering to harvesting time, hand pollination (HP) was done by gently rubbing the heads with the help of muslin cloth at flowering duration and sib-pollination (SIP) was done by collecting pollen from males plants of the same genotype and mixed to dust on sunflower heads of receiver female plants of the same one.

Each genotype was sown, after harvesting wheat in both seasons, in five ridges with a ridge length of 4 m spaced at 60 cm between ridges and 30 cm apart. Tested sunflower genotypes as open pollinated genotypes i.e. L125, Sakha$_{53,}$ L235, L880, L770, L92, L120, L167, L460 and L355 were received from Oil Crops Research Department, Field Crop Research Institute, Agricultural Research Center, Egypt.

2.3 AGRICULTURAL PRACTICES

Sunflower genotypes seeds were hand-planted on ridges, 60 cm as well as 30 cm apart between hills, during the first week of June in both seasons. Plants were thinned at 15 days after sowing to secure one plant per hill. Fertilizer was added in two equal portions prior to the first and the second irrigations in the form of urea (46.6 % N). All other cultural practices were applied as recommended.

2.4 DATA COLLECTED

At harvest, five guarded plants were randomly selected from the 2nd and 4th ridges, harvested, tied and left to dry to determine yield and yield attributes viz., head diameter, number of filled seeds per head, number of unfilled seeds per head, total number of seeds per head, 100-seed mass and seed mass per plant. The seed set percent was calculated by the formula given by Roath and Miller (1982) as, seed setting percentage = (number of filled seeds/ total seeds) × 100. Autogamy and self-compatibility percent was calculated by formulae given by George et al. (1980) as, autogamy % = percent seed set under self-pollination/ percent seed set under open pollination. Self-compatibility % = percent seed set under hand pollination/ percent seed set under open pollination. The mass of hulls and kernels recorded by drawing 100-seeds at random, and then their kernels and hulls were separated to record the mass of the separated hundred hulls and kernels. Hull percent was calculated by the following formula, Hull % = (Mass of hull / Total mass of seeds) × 100. Kernel to hull (K/H) ratio was calculated by dividing the kernel mass of hundred seed by their hull mass. Plants of central ridge from each plot were harvested for determining seed yield per m^2 and converted to seed yield in kg per fed. Seed oil content was determined according to AOAC, (1990).

2.5 STATISTICAL ANALYSIS

Analysis of variance according to Gomez and Gomez (1984) for each variable was done using randomized complete block design for all studied traits. Combined analysis of variance across the two seasons was done after confirmation of homogenous (Table 1) for all studied traits using F. max test method of Hartley (1950).

Genotypic and phenotypic coefficients of variation (Burton and DeVane 1953), estimation of broad sense heritability (H^2_b) (Hansen et al., 1956), genetic advance as percent of the mean (Johnson et al., 1955) were estimated. Phenotypic (r_p) and genotypic (r_g) correlation coefficients between two traits such as X and Y were calculated using formula of Weber and Moorthy (1952): $r_p = Cov_p (X, Y) / (V_p (X) \times V_p (Y))^{0.5}$, where Cov P (X, Y) = Phenotypic covariance of traits X and Y. Vp (X) and VP (Y) = Phenotypic variance of trait X and Y, respectively. And $r_g = Cov_g (X, Y) / (V_g (X) \times V_g (Y))^{0.5}$, where Cov_g (X, Y) = Genotypic covariance of traits X and Y. V_g (X) and V_p (Y) = Genotypic variance of trait X and Y, respectively. Moreover, phenotypic and genotypic path analysis (Dewey and Lu 1959) was also done.

Table 1: Seasonal Hartley tests (F. max) of error variances for all studied traits

Item	head diameter	Number of filled seeds per head	Number of unfilled seeds per head	Total number of seeds per head	Seed setting percentage	100.seed mass	kernel mass
F. max	1.46	1.13	1.07	1.08	1.07	1.24	1.03
F. tabulated	1.48	1.48	1.48	1.48	1.48	1.48	1.48
Significance	NS	NS	NS	NS	NS	NS	NS
Item	Hull mass	Hull content	Kernel to hull ratio	Seed mass per plant	Seed yield per fed	Seed oil content	
F. max	1.18	1.24	1.12	1.24	1.11	1.43	
F. tabulated	1.48	1.48	1.48	1.48	1.48	1.48	
Significance	NS	NS	NS	NS	NS	NS	

3 RESULTS AND DISCUSSION

3.1 ANALYSIS OF VARIANCE

The combined analyses of variance for all studied traits of 10 sunflower genotypes over years and pollination methods are presented in Table 2. In this respect, the main effects of years, pollination methods and genotypes were highly significant for all studied traits, indicating that significant effect of these main effects on the performance of all studied traits. The interactions of year with pollination methods and with genotypes had insignificant effect on the performance of all studied traits. This indicated that the pollination methods and tested sunflower genotypes had the same ranks from year to another. Moreover, the insignificant triple interaction among year, pollination methods and genotypes was observed for all studied traits. This indicated that climatic conditions of two years had the same effect on pollination methods and tested sunflower genotypes, hence it was ignored in discussion. However, the interaction of pollination methods with tested sunflower genotypes was highly significant for all studied traits. This indicated that tested sunflower genotypes significantly differed from pollination method to another for all studied traits, hence it was considered as important interaction to discussion. Similar significant interactive effect of pollination methods with sunflower genotypes were reported by Supriya et al. (2016), Neelima et al. (2016) and Supriya et al. (2017).

3.2 POLLINATION METHODS EFFECTS

The results of pollination methods in Table (3) revealed that there were significant differences between various pollination treatments for all studied traits. Similar significant effects of pollination methods were reported by Talele (2011) and Osman and Siham (2015).

As shown in the combined analysis, obviously, open pollination gave the highest values for head diameter, number of filled seeds per head, percentage of seed setting, kernel to hull ratio, seed mass per plant, seed yield per fed and seed oil content. It exceeds self-pollination by 25.10 %, 41.45 %, 44.03 %, 3.55 %, 56.68 %, 57.25 %, 15.16 %, respectively. This may be due to the effective transfer of foreign pollens by pollinating insects to the

Table 2: Combined analysis of variance for all studied traits of 10 sunflower genotypes over two years and four pollination methods

SOV	df	Head diameter	Number of filled seeds per head	Number of unfilled seeds per head	Total number of seeds per head	Seed setting percentage	100.seed mass	Kernel mass
Year (Y)	1	**	**	**	**	*	**	**
Pollination methods (P)	3	**	**	**	**	**	**	**
Y x P	3	NS	NS	NS	NS	NS	NS	NS
Reps in Y x P	2	NS	NS	NS	NS	NS	NS	NS
Genotypes (G)	9	**	**	**	**	**	**	**
P x G	27	**	**	**	**	**	**	**
Y x G	9	NS	NS	NS	NS	NS	NS	NS
Y x P x G	27	NS	NS	NS	NS	NS	NS	NS
Error	237							

SOV	df	Hull mass	Hull content	Kernel to hull ratio	Seed mass per plant	Seed yield per fed	Seed oil content
Year (Y)	1	**	**	**	**	**	**
Pollination methods (P)	3	**	**	**	**	**	**
Y x P	3	NS	NS	NS	NS	NS	NS
Reps in Y x P	2	NS	NS	NS	NS	NS	NS
Genotypes (G)	9	**	**	**	**	**	**
P x G	27	**	**	**	**	**	**
Y x G	9	NS	NS	NS	NS	NS	NS
Y x P x G	27	NS	NS	NS	NS	NS	NS
Error	237						

Table 3: Comparison of means of pollination methods for all studied traits across 2018 and 2019 seasons

Pollination methods	Head diameter (cm)	Number of filled seeds per head	Number of unfilled seeds per head	Total number of seeds per head	Seed setting percentage	100-Seed mass (g)	Kernel mass (mg)
Open	19.54	471.92	101.99	573.91	81.52	6.07	483.92
Selfing	14.63	276.32	316.72	593.03	45.63	6.66	552.33
Hand	17.48	340.78	201.15	541.93	62.00	6.52	535.63
Sibbing	18.95	444.22	132.72	576.93	76.41	6.26	505.03
$LSD_{5\%}$	0.24	1.55	1.55	2.28	0.21	0.05	1.46

Pollination methods	Hull mass (mg)	Hull content (%)	Kernel to hull ratio	Seed mass per plant (g)	Seed yield per fed (kg)	Seed oil content (%)
Open	172.99	0.39	2.80	42.62	1112.22	42.08
Selfing	204.40	0.89	2.70	18.46	475.44	35.70
Hand	189.05	0.60	2.84	25.27	773.90	37.49
Sibbing	183.71	0.44	2.75	35.42	953.64	39.96
$LSD_{5\%}$	1.20	0.01	0.02	0.51	10.38	0.36

receptive stigma, thereby increasing percentage of seed setting and gave higher number of filled seeds per head. The reverse was true, with selfing, which pollination had the highest values of number of unfilled seeds per head, total number of seeds per head, 100-seed mass (g), kernel mass (mg), hull mass (mg) and percentage of hull content. It exceeds open pollination by 210.53 %, 3.33 %, 9.78 %, 14.14 %, 18.16 %and 129.63 % in the respective order. Other methods, hand and sib-pollination had the moderate effect on most of studied traits.

3.3 GENOTYPIC EFFECTS

It is apparent from the pooled data in Table (4) that significant differences were detected among the ten tested sunflower genotypes for all studied traits, indicated that there is valuable genetic variation in these genotypes to warrant selection for improvement sunflower crop. Similar significant genotypic effects for all studied traits were reported by Supriya et al. (2016), Neelima et al. (2016) and Supriya et al. (2017). Irrespective of pollination methods imposed, 'Sakha 53' (21.55 cm) followed by 'L120' (19.83 cm) behaved as the largest head diameter; 'L120' (534.95) followed by 'L880' (503.37) gave more filled seeds per head; 'L120' (80.07 %) followed by 'L92' (76.59 %) had the highest proportion of seed setting; 'L355' (6.76 g) followed by 'L167' (6.73 g) possessed the biggest mass of 100-seed; 'L355' (538.58 mg) followed by ''L880 (535.33 mg) had the biggest mass of kernel; 'L880' (199.71 mg) followed by 'L355' (198.61 mg) expressed the highest mass of hull; the highest proportion of hull was detected in 'L355' (1.07 %) followed by 'L167' (0.82 %); 'L120' (2.98 %, 37.28 g, 1022.95 kg and 42.86 %) followed by 'L92' (2.83 %, 33.83 g, 890.05 kg and 40.21 %) behaved as the largest ratio of Kernel to hull, seed mass per plant, seed yield per fed and seed oil content, respectively. Hence, these genotypes are the best stock for improving sunflower crop in terms of most studied traits.

3.4 PERCENTAGE OF AUTOGAMY AND SELF-COMPATIBILITY

A genotype is considered self-fertile if the maximum percentage of seed setting takes place under self-pollination. Among the ten sunflower genotypes as shown in Fig. (1), L120 recorded the highest proportion of autogamy on one hand (0.664 %) followed by L92 (0.636 %) and L880 (0.618 %). On the other hand, L120 (0.839 %), L880 (0.807 %) and L92 (0.806 %) behaved as self-compatible sunflower genotypes. This may be due to these genotypes possessed a high number of florets per unit area, a random orientation of stigmas, and the stigma receptive surface vertically presented. Thus, these genotypes can be used in sunflower breeding program to increasing proportion of self-compatibility.

3.5 INTERACTION EFFECTS

The interactive effect of pollination methods with ten sunflower genotypes was significant for all studied traits, as combined analysis shown in Table (5), indicated that traits of sunflower genotypes especially yield and yield-components varied according to pollination meth-

Table 4: Comparison of means of ten sunflower genotypes for all studied traits across 2018 and 2019 seasons

Sunflower genotypes	Head diameter (cm)	Number of filled seeds per head	Number of unfilled seeds per head	Total number of seeds per head	Seed setting percentage	100-Seed mass (g)	Kernel mass (mg)
L125	16.45	349.12	173.17	522.28	66.82	6.55	522.50
Sakha 53	21.55	379.45	206.02	585.47	64.66	6.04	506.42
L235	17.21	376.61	171.39	548.00	69.05	6.51	518.21
L880	18.60	503.37	198.52	701.89	71.99	6.16	535.33
L770	16.08	321.95	180.00	501.95	64.32	6.59	528.33
L92	18.26	452.70	139.17	591.87	76.59	6.28	506.75
L120	19.83	534.95	131.83	666.78	80.07	5.70	492.83
L167	15.79	264.95	245.50	510.45	51.97	6.73	532.50
L460	17.68	420.20	154.50	574.70	73.23	6.42	510.83
L355	15.06	229.78	281.33	511.12	45.19	6.76	538.58
LSD 5%	0.37	2.45	2.46	3.60	0.33	0.07	2.31

Sunflower genotypes	Hull mass (mg)	Hull content (%)	Kernel to hull ratio	Seed mass per plant (g)	Seed yield per fed (kg)	Seed oil content (%)
L125	188.58	0.58	2.77	30.96	795.79	38.32
Sakha$_{53}$	190.30	0.54	2.66	28.48	849.66	40.06
L235	186.70	0.52	2.78	31.54	822.01	39.14
L880	199.71	0.41	2.68	28.45	824.84	38.48
L770	190.43	0.64	2.78	29.61	770.06	37.77
L92	178.80	0.41	2.83	33.83	890.05	40.21
L120	166.05	0.32	2.98	37.28	1022.95	42.86
L167	191.89	0.82	2.78	26.75	746.59	36.51
L460	184.30	0.46	2.78	32.36	842.15	39.69
L355	198.61	1.07	2.71	25.14	723.89	35.04
LSD 5%	1.90	0.02	0.03	0.81	16.42	0.57

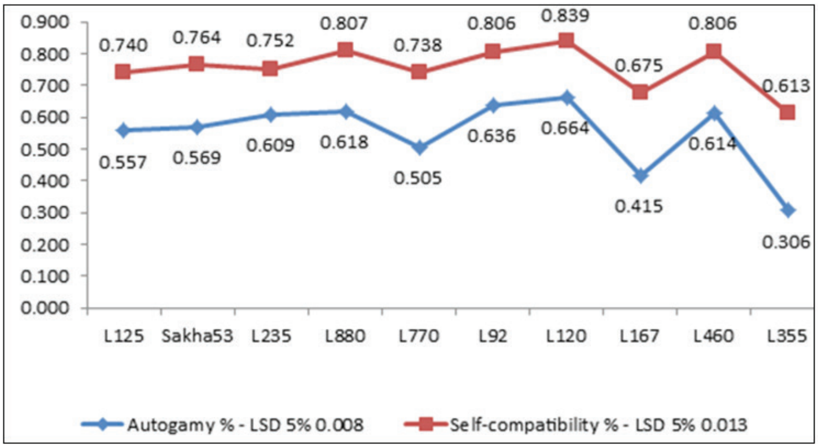


Figure 1: Autogamy percentage and self-compatibility percentage of ten sunflower genotypes across 2018 and 2019 summer seasons

Table 5: Comparison of means of pollination methods interaction with ten sunflower genotypes across 2018 and 2019 summer seasons

Interaction	Head diameter (cm)	Number of filled seeds per head	Number of un-filled seeds per head	Total number of seeds per head	Seed setting percentage	100-seed mass (g)	Kernel mass (mg)
OP x L125	17.67	428.12	93.25	521.37	82.12	6.20	489.83
OP x Sakha 53	25.67	459.78	125.33	585.12	78.58	5.72	467.50
OP x L235	18.63	449.10	88.81	537.91	83.49	6.18	478.33
OP x L880	20.10	608.12	95.68	703.79	86.41	5.97	507.50
OP x L770	17.42	422.45	97.92	520.37	81.18	6.21	494.17
OP x L92	19.87	534.45	58.93	593.38	90.07	6.02	473.83
OP x L120	22.35	626.45	50.92	677.37	92.49	5.55	458.83
OP x L167	17.38	356.45	155.58	512.03	69.61	6.35	496.17
OP x L460	19.10	517.45	77.92	595.37	86.91	6.08	475.50
OP x L355	17.17	316.78	175.58	492.37	64.34	6.38	497.50
SP x L125	13.67	247.12	293.25	540.37	45.73	6.81	551.17
SP x Sakha 53	17.88	266.12	328.92	595.03	44.72	6.41	540.83
SP x L235	14.47	302.12	292.25	594.37	50.83	6.76	548.50
SP x L880	15.53	394.12	344.25	738.37	53.38	6.37	567.50
SP x L770	13.23	214.12	308.58	522.70	40.96	6.90	565.17
SP x L92	15.38	356.12	265.58	621.70	57.28	6.54	538.50
SP x L120	17.10	409.12	257.25	666.37	61.41	6.01	523.83
SP x L167	12.87	152.12	374.25	526.37	28.90	7.04	567.50
SP x L460	14.73	318.12	277.58	595.70	53.40	6.71	545.50
SP x L355	11.45	104.12	425.25	529.37	19.66	7.07	574.83
HP x L125	16.68	295.12	190.58	485.70	60.76	6.65	536.17
HP x Sakha 53	20.07	325.78	216.58	542.37	60.07	6.19	531.83
HP x L235	17.40	319.12	189.25	508.37	62.77	6.63	535.17
HP x L880	18.55	478.12	207.92	686.03	69.69	6.64	549.17
HP x L770	16.03	291.12	194.58	485.70	59.94	6.68	539.17
HP x L92	18.17	397.12	149.92	547.03	72.60	6.43	529.17
HP x L120	19.33	491.12	141.92	633.03	77.59	5.79	515.83
HP x L167	15.77	228.12	257.25	485.37	47.00	6.81	541.50
HP x L460	17.90	388.12	165.58	553.70	70.09	6.53	533.17
HP x L1250	14.93	194.12	297.92	492.03	39.45	6.84	545.17
SIP x L355	17.80	426.12	115.58	541.70	78.66	6.55	512.83
SIP x Sakha 53	22.57	466.12	153.25	619.37	75.26	5.86	485.50
SIP x L235	18.33	436.12	115.25	551.37	79.10	6.49	510.83
SIP x L880	20.20	533.12	146.25	679.37	78.47	5.64	517.17
SIP x L770	17.63	360.12	118.92	479.03	75.18	6.59	514.83
SIP x L92	19.63	523.12	82.25	605.37	86.41	6.13	485.50
SIP x L120	20.53	613.12	77.25	690.37	88.81	5.46	472.83
SIP x L167	17.13	323.12	194.92	518.03	62.37	6.72	524.83
SIP x L460	19.00	457.12	96.92	554.03	82.51	6.37	489.17
SIP x L355	16.70	304.12	226.58	530.70	57.30	6.75	536.83
LSD 5%	0.74	4.90	4.91	7.20	0.66	0.15	4.61

OP: open pollination, SP: self-pollination, HP: hand-pollination and SIP: sib-pollination

Table 5: Continued

Interaction	Hull mass (mg)	Hull content (%)	Kernel to hull ratio	Seed mass per plant (g)	Seed yield per fed (kg)	Seed oil content (%)
OP x L125	174.97	0.41	2.80	42.77	1058.32	41.60
OP x Sakha 53	171.23	0.37	2.73	41.51	1183.29	42.79
OP x L235	170.52	0.38	2.81	43.54	1103.32	43.12
OP x L880	180.80	0.30	2.81	39.64	1147.50	41.72
OP x L770	178.63	0.42	2.77	42.17	1025.86	40.25
OP x L92	164.73	0.31	2.88	46.50	1176.32	44.11
OP x L120	149.53	0.24	3.07	48.98	1352.95	45.15
OP x L167	180.80	0.51	2.75	39.50	995.90	39.36
OP x L460	167.60	0.32	2.84	43.72	1119.19	43.56
OP x L355	191.07	0.60	2.60	37.86	959.51	39.13
SP x L125	208.00	0.84	2.65	19.20	466.24	34.72
SP x Sakha 53	209.67	0.79	2.58	17.11	466.93	38.24
SP x L235	206.80	0.68	2.65	19.80	479.53	35.64
SP x L880	209.20	0.53	2.71	14.65	404.00	35.57
SP x L770	208.93	0.98	2.71	17.18	446.16	34.67
SP x L92	191.11	0.54	2.82	21.17	493.70	36.61
SP x L120	185.13	0.45	2.83	24.20	642.11	42.00
SP x L167	209.67	1.38	2.71	16.06	441.73	33.05
SP x L460	203.53	0.64	2.68	20.09	484.64	35.86
SP x L355	212.00	2.04	2.71	15.17	429.33	30.61
HP x L125	186.67	0.63	2.87	25.48	724.45	37.18
HP x Sakha 53	197.47	0.61	2.69	24.64	794.77	39.55
HP x L235	185.93	0.58	2.88	26.11	761.40	37.69
HP x L880	210.33	0.44	2.61	25.82	799.93	37.92
HP x L770	187.17	0.64	2.88	24.06	695.90	36.77
HP x L92	183.90	0.46	2.88	29.20	840.51	39.24
HP x L120	171.03	0.35	3.02	34.16	974.37	41.20
HP x L167	188.68	0.83	2.87	18.68	685.58	34.52
HP x L460	185.33	0.48	2.88	27.62	785.18	38.87
HP x L1250	193.95	1.00	2.81	16.93	676.94	31.98
SIP x L355	184.70	0.43	2.78	36.41	934.14	39.78
SIP x Sakha 53	182.83	0.39	2.66	30.67	953.63	39.66
SIP x L235	183.57	0.42	2.78	36.72	943.80	40.12
SIP x L880	198.50	0.37	2.61	33.70	947.93	38.72
SIP x L770	187.00	0.52	2.75	35.02	912.33	39.38
SIP x L92	175.47	0.34	2.77	38.46	1049.67	40.88
SIP x L120	158.50	0.26	2.98	41.79	1122.37	43.09
SIP x L167	188.40	0.58	2.79	32.77	863.13	39.09
SIP x L460	180.73	0.40	2.71	38.02	979.57	40.47
SIP x L355	197.43	0.65	2.72	30.59	829.78	38.43
LSD 5%	3.79	0.03	0.07	1.61	32.83	1.13

OP: open pollination, SP: self-pollination, HP: hand-pollination and SIP: sib-pollination ods. Similar significant interactive effect of pollination methods with sunflower genotypes were reported by Supriya et al. (2016), Neelima et al. (2016) and Supriya et al. (2017). As shown in pooled data of pollination methods interaction with ten sunflower genotypes, the largest head diameter (25.67) was recorded by 'Sakha 53' under open pollination, the highest number of filled seeds per head (626.45), percentage of seed setting (92.49 %), kernel to hull ratio (3.07), seed mass per plant (48.98 g), seed yield per fed (1352.95 kg) and seed oil content (45.15 %) was recorded by 'L120' under open pollination. Conversely, the biggest mass of 100-seed (7.07), kernel (574.83), hull (212.00) and hull content (2.04) was detected in 'L355' with self-pollination. This indicated that possibility selection of genotypes on pollination methods, where performance of genotypes varied according to pollination method used.

3.6 GENETIC VARIABILITY

To have better understanding of the extent of genetic variability for the ten sunflower genotypes, phenotypic (PCV)and genotypic (GCV) coefficient of variation, heritability in broad sense (H^2b) and expected genetic advance as percent mean were computed for all studied traits as presented in Table (6). Phenotypic coefficient of variation (PCV) was slightly higher than genotypic coefficient of variation (GCV) for all studied traits, indicating negligible influence of environmental conditions on the expression of all traits. Consequently, the selection would be effective to genetic improvement of the studied traits for these materials. High PCV and GCV as shown in combined analysis, were observed for head diameter, number of filled seeds per head, number of unfilled seeds per head, total number of seeds per head, percentage of seed setting, percentage of hull content , seed mass per plant and seed yield per fed, and dropped to moderate for 100-seed mass, hull mass and seed oil content, while drastically reduced for kernel mass and kernel to hull ratio. High heritability in broad sense was recorded for all studied traits, indicates a genotype can be identified by its phenotypic performance, thus making the selection more effective for improving these traits. That has been observed in earlier studies by Neelima et al. (2012), Tyagi and Khan (2013), Deengra et al. (2013) and Sanju et al. (2018).

Heritability in broad sense along with expected genetic advance as percent mean, as shown in Table (6), is normally more useful in predicting the genetic gain under selection than heritability estimates alone as confirmed by Johnson et al. (1955). High values of heritability coupled with high values of genetic advance percentage (more than 20 %) were detected for head diameter, number of filled seeds per head, number of unfilled seeds per head, total number of seeds per head, percentage of seed setting , hull content percentage, seed mass per plant and seed yield per fed, indicating the importance of additive gene effects in the inheritance of these traits, thus, selection for these traits would be effective for improving seed mass per plant. High heritability coupled with moderate expected percentage of genetic advance (10-20 %) were recorded for 100-seed mass, hull mass and seed oil content, indicated that these traits appear to be controlled by both additive and non-additive gene action. However high heritability coupled with low expected percentage of

Table 6: Pooled analysis of genetic parameters for all studied traits across 2018 and 2019 seasons

Genetic parameter	Head diameter	Number of filled seeds per head	Number of unfilled seeds per head	Total number of seeds per head	Seed setting percentage	100-Seed mass	Kernel mass
GCV	11.10	25.43	24.75	11.84	16.12	5.10	2.80
PCV	11.24	25.53	24.85	11.94	16.20	5.20	2.87
H^2 bs	97.40	99.25	99.22	98.43	98.91	96.17	95.55
GV %	22.56	52.19	50.79	24.21	33.02	10.31	5.64
Genetic parameter	**Hull mass**	**Hull content**	**Kernel to hull ratio**	**Seed mass per plant**	**Seed yield per fed**	**Seed oil content**	
GCV	5.07	35.30	2.92	11.43	10.00	5.38	
PCV	5.20	38.67	3.14	11.68	10.21	5.55	
H^2 bs	94.87	83.33	86.58	95.86	95.93	93.83	
GV %	10.16	66.39	5.59	23.06	20.17	10.73	

GCV: Genotypic coefficient of variation, PCV: Phenotypic coefficient of variation, H2bs: Broad-sense heritability and GV %: Expected genetic advance as percent of mean genetic advance (less than 10 %) were recorded for kernel mass and kernel to hull ratio, indicated that an increased influence of environmental conditions on this character. Thus selection procedure involving progeny testing are recommended for this trait.

3.7 ASSOCIATION OF TRAITS AND PATH ANALYSIS (AS SELECTION CRITERIA)

Phenotypic and genotypic correlation were estimated between seed mass per plant and its attributes for the 10 sunflower genotypes based on average of 2018 and 2019 summer seasons as presented in Table (7). Seed mass per plant was positively and significant or highly significant correlated with head diameter, number of filled seeds per head, total number of seeds per head, percentage of seed setting and kernel to hull ratio at phenotypic and genotypic levels, indicating that selection will be more effective for the largest head diameter, more of filled seeds, the highest proportion of seed setting and kernel to hull ratio. These findings are confirmed by those of Tyagi and Khan (2013), Deengra et al. (2013) and Sanju et al. (2018). Furthermore, positive and significant or highly significant phenotypic and genotypic correlation was detected among the previous traits i.e. head diameter, number of filled seeds per head, total number of seeds per head, percentage of seed setting and kernel to hull ratio at phenotypic and genotypic levels, indicated that selection practiced for any one of the previous traits would improve the other ones, especially seed mass per plant. Therefore, these traits could be considered as indicators to achieve desirable genetic improvement for seed mass per plant of sunflower.

For further clarification about interrelationships between seed mass per plant and its attributes, the phenotypic and genotypic path analysis divided phenotypic and genotypic correlation into direct and joint effects, where seed mass per plant was considered a dependent variable and yield attributes were independent variables as shown in Table (8) and Fig.(2).

A critical perusal of phenotypic and genotypic path analysis revealed that seed setting percentage had the highest positive direct effects (p = 0.598, G = 1.759) on seed mass per plant, followed by kernel mass only at genotypic level (1.122). The highest positive direct effects of the two previous mentioned traits in addition to their highly significant correlation coefficient with seed mass per plant indicated that the direct selection through these two traits would be effective for sunflower improvement.

Conversely, the direct effects of head diameter, number of filled seeds per head, 100-seed mass and hull mass on seed mass per plant were negative and negligible, although their phenotypic and genotypic correlation with seed mass per plant were positive and highly significant. Consequently, the indirect effects of the previous mentioned traits would be more effective to improve seed mass per plant of these materials than direct ones. The highest positive phenotypic and genotypic indirect effects on seed mass per plant were detected for head diameter (p = 0.367, G = 1.102) through seed setting percentage followed by 100-seed mass (p = 0.303, G = 1.232), kernel mass only at phenotypic level (0.322) and hull mass (p = 0.081, G = 0.458); number of filled seeds per head *via* seed setting percentage (p = 0.554, G =

Table 7: The pooled phenotypic (above diagonal) and genotypic (below diagonal) correlations of seed mass per plant and its attributes across 2018 and 2019 summer seasons

Traits	HD	FS	UFS	TS	SS	HSM	KM	HM	HC	KHR	SMP
HD	1	0.70**	-0.46**	0.69**	0.61**	-0.89**	-0.73**	-0.41**	-0.70**	0.07	0.45**
FS	0.72**	1	-0.78**	0.90**	0.93**	-0.87**	-0.64**	-0.58**	-0.93**	0.44**	0.76**
UFS	-0.47**	-0.78**	1	-0.43**	-0.95**	0.59**	0.76**	0.78**	0.90**	-0.63**	-0.93**
TS	0.71**	0.90**	-0.44**	1	0.68**	-0.84**	-0.40*	-0.30	-0.72**	0.20	0.46**
SS	0.63**	0.93**	-0.96**	0.68**	1	-0.74**	-0.73**	-0.69**	-0.98**	0.52**	0.88**
HSW	-0.92**	-0.89**	0.61**	-0.87**	-0.76**	1	0.76**	0.62**	0.77**	-0.41**	-0.66**
KW	-0.74**	-0.66**	0.78**	-0.41**	-0.76**	0.78**	1	0.89**	0.70**	-0.63**	-0.85**
HW	-0.42**	-0.59**	0.81**	-0.30	-0.72**	0.64**	0.92**	1	0.61**	-0.91**	-0.93**
HC	-0.76**	-1.01**	0.98**	-0.78**	-1.04**	0.86**	0.78**	0.70**	1	-0.41**	-0.81**
KHR	0.08	0.47**	-0.70**	0.20	0.58**	-0.45**	-0.71**	-0.92**	-0.53**	1	0.83**
SMP	0.46**	0.79**	-0.96**	0.48**	0.91**	-0.70**	-0.91**	-1.01**	-0.93**	0.94**	1

HD-head diameter, FS-number of filled seeds per head, UNS-number of filled seeds per head, TS-Total number of seeds per head, SS-seed setting percentage, HSM-100-seed mass, KM-kernel mass, HM-hull mass, HC-hull content, KHR-kernel to hull ratio and SMP-seed mass per plant

Table 8: Pooled phenotypic (P) and genotypic (G) path analysis of seed mass per plant and its attributes traits across 2018 and 2019 summer seasons

Traits		P1	P2	P3	P4	P5	P6	Correlation
Head diameter (P1)	P	-0.571	-0.056	0.367	0.303	0.322	0.081	0.446
	G	-0.216	-1.279	1.102	1.232	-0.834	0.458	0.462
Filled seeds per head (P2)	P	-0.402	-0.080	0.554	0.294	0.285	0.113	0.763
	G	-0.155	-1.786	1.637	1.188	-0.738	0.640	0.787
Seed setting percentage (P3)	P	-0.351	-0.074	0.598	0.251	0.326	0.134	0.884
	G	-0.135	-1.662	1.759	1.018	-0.848	0.774	0.906
100.seed mass (P4)	P	0.510	0.069	-0.443	-0.339	-0.339	-0.121	-0.662
	G	0.200	1.589	-1.342	-1.334	0.874	-0.689	-0.703
Kernel mass (P5)	P	0.414	0.051	-0.438	-0.258	-0.445	-0.174	-0.850
	G	0.161	1.175	-1.329	-1.040	1.122	-0.997	-0.909
Hull mass (P6)	P	0.237	0.046	-0.410	-0.210	-0.397	-0.195	-0.930
	G	0.092	1.059	-1.262	-0.853	1.037	-1.079	-1.006
Residual effect	P	0.057						
	G	0.305						

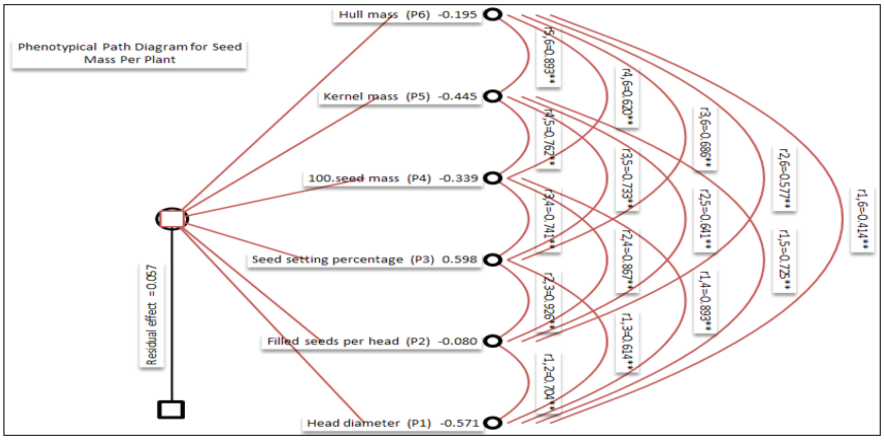


Figure 2a: Phenotypic path diagram for seed mass per plant

1.637), followed by 100-seed mass ($p = 0.294$, G = 1.188), kernel mass only at phenotypic level (0.285) and hull mass ($p = 0.113$, G = 0.640); seed setting percentage via 100-seed mass ($p = 0.251$, G = 1.018), followed by kernel mass at only phenotypic level (0.326) and hull mass ($p = 0.134$, G = 0.774); 100-seed mass ($p = 0.510$, G = 0.200) followed by number of filled seeds per head ($p = 0.069$, G = 1.589) followed kernel mass at only genotypic level (0.874); kernel mass via head diameter ($p = 0.414$, G = 0.161) followed number of filled seeds per head ($p = 0.051$, G = 1.175) and hull mass via head diameter ($p = 0.237$, G = 0.092) followed by number of filled seeds per head($p = 0.046$, G = 1.059).

It is apparent from the above-mentioned results, it can be concluded that preferred improvement of seed mass per plant may be achieved through selecting genotypes having the biggest head diameter, more number of filled seeds per head, the highest proportion of seed setting and the heaviest seed, kernel and hull mass. These results agreed with those of Tyagi and Khan (2013), Deengra et al. (2013) and Sanju et al. (2018).

The residual effect being 0.057 and 0.305 at phenotypic and genotypic levels, respectively indicated that independent traits which are included at the phenotypic and genotypic path analysis, explained 94.3 and 69.5 % of the total variation, respectively in seed mass per plant. The highest residual effects of phenotypic and genotypic path analyses, indicated that the presence of other traits that are not included in the present study were associated with the highest effect on seed mass per plant.

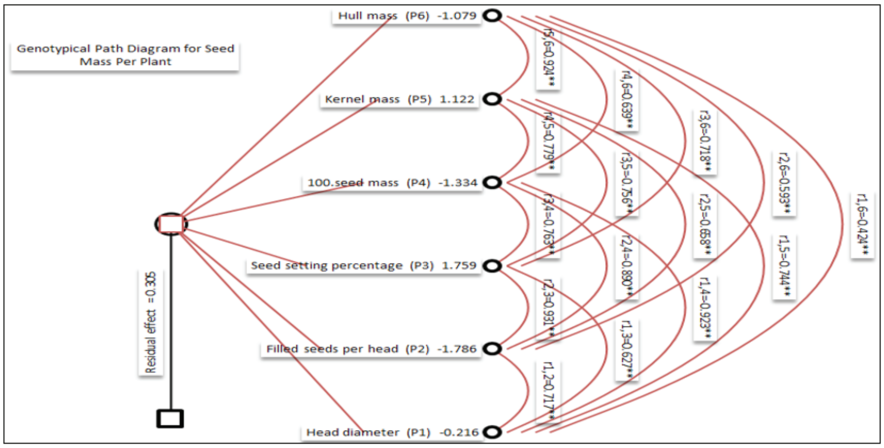


Figure 2b: Genotypic path diagram for seed mass per plant

4 CONCLUSION

From above-mentioned results, it can be concluded that open pollination followed by sibbing one was superior to other pollination methods and leading to high seed mass per plant and higher accumulation of oil content. Higher seed yield was achieved due to high seed set percent, number of filled seeds per head, high kernel to hull ratio and higher kernel proportion in seed. Genotypes L120 followed by L92 and L880 behaved as self-compatible sunflower genotypes**.** Selection would be more effective to improve seed mass per plant through selecting genotypes having the largest head diameter, more number of filled seeds per head, the highest proportion of seed setting and the heaviest seed, kernel and hull mass as proven by high phenotypic and genotypic coefficient of variation and high heritability coupled with high genetic advance (as % of mean) as well as correlation and path analyses at both phenotypic and genotypic levels.

5 ACKNOWLEDGEMENTS

This study was funded by Oil Crops Research Department, Field Crops Research Institute, Agricultural Research Center, Egypt. We thank the director of Kafr-El-Hamam Agricultural research Station, Sharkia Governorate, ARC, Egypt for providing all help to complete this work.

6 REFERENCES

AOAC. (1990). *Official methods of analysis*. 15th ed., Association of official analytical chemists, Virginia, USA; p. 770–1.

Baraiya, V. K.; Jagtap, P.K.; Himalay, R.P. (2018). Genetic variability, heritability and genetic advance for seed yield in sunflower (*Helianthus annuus* L.). *International Journal of Chemical Studies*, *6*(5), 2141-2143.

Beg, A.; Aslam, M.; Pirvu, N.; Khan, N.A.; Mirza, M.S. (1987). Self-compatibility in different sunflower genotypes in Pakistan. *Helia*, *10,* 39-41.

Burton, G. W.; DeVane, E. H. (1953). Estimating heritability in tall fescue (*Festuca arundinacea*) from replicated clonal material. *Agronomy Journal*, *45*(10), 478–481. https://doi.org/10.2134/agronj1953.00021962004500100005x

Deengra, S. K.; Kumar, N.; Kumar, V.; Dhaka, R. P. S. (2013). Correlation and path coefficient analysis in Sunflower (*Helianthus annuus* L.). *Progressive Agriculture*, *13*(1), 197-202.

Dewey, D.R.; Lu, K.H. A (1959). *Correlation and path coefficient analysis of components of crested wheat grass seed production. Agronomy Journal,* 51, 515 – 518. https://doi.org/10.2134/agronj1959.00021962005100090002x

Dudhe, M. Y.; Rajguru, A. B.; Bhoite, K. D.; Madhuri, P. (2017). Genetic evaluation and identification of stable sunflower genotypes under semi-arid dryland conditions of Telangana and Maharashtra state sabrao. *Journal of breeding and genetics*, *49*(1), 83-93.

George, D l; Shein, S E; Knowles, P.F. (1980). Compatibility, autogamy and environmental effects on seed set in selected sunflower hybrids, and their inbred parents. *Abstracts of 9th International conference Malagu, Spain 14*.

Gomez, K.A.; Gomez, A.A. (1984). *Statistical Procedures for Agricultural Research*. 2nd Edition, John Wiley and sons Inc., Hoboken, p. 680.

Hansen, W. D.; Robinson, H. F.; Comstock, R. E. (1956). Biometric studies of yield in segregating populations of Korean lespedeza. *Agronomy Journal*, *48*, 268-272. https://doi.org/10.2134/agronj1956.00021962004800060008x

Hartley, H.O. (1950). The use of range in analysis of variance. *Biometrika*, *37*, 271-280. https://doi.org/10.1093/biomet/37.3-4.271

Johnson, H. W.; Robinson, H. F.; Comstock, R. E. (1955). Estimates of genetic and environmental variability in Soybean.

Agronomy Journal, *47,* 314-318. https://doi.org/10.2134/agronj1955.00021962004700070009x

Kalukhe V. K.; Moon, M. K.; Magar, N. M.; Patil, S. S. (2010). Genetic variability in sunflower (*Helianthus annuus* L.). *Bioinfolet, 7*(3), 197-200.

Khan, H. ; Muhammad, S. (2007). Genetic analysis of yield and some yield components in sunflower. *Sarhad Journal of Agriculture, 23*(4), 985-990.

Neelima, S.; Kumar, K. A., Venkataramanamma, K.; Padmalatha, Y. (2016). Genetic variability and genetic diversity in sunflower. *Electronic Journal of Plant Breeding, 7*(3), 703-707.

Neelima, S.; Parameshwarappa, K. G.; Kumar, Y. P. (2012). Association and path analysis for seed yield and component characters in Sunflower (*Helianthus annuus* L.). *Electronic Journal of Plant Breeding, 3*(2), 716-721. https://doi.org/10.5958/0975-928X.2016.00090.9

Osman, A.A.; Siham, K. Ab. N. (2015). Efficacy of honeybees *(Apis mellifera)* on the production of sunflower (*Helianthus annuus* L.) seeds in the Sudan. *Journal of Experimental Biology and Agricultural Sciences, 3*(2),191:195. https://doi.org/10.18006/2015.3(2).191.195

Prabhakaran, V.; Reddy, S. M.; Reddy, T. D.; Ranganatha, A. R. G. (2013). Character association and path analysis in sunflower (*Helianthus annuus* L.). *Journal of Oilseeds Research, 30*(1), 65-67.

Roath, W W; Miller, J F. (1982). Environmental effects on seed set in oilseed Sunflower (*Helianthus annuus* L.). *Canadian Journal of Plant Sciences, 62*, 867– 873. https://doi.org/10.4141/cjps82-130

Sameena, S. (2003). *Effect of pollen blend on seed yield and quality of sunflower (Helianthus annuus L.*). M.Sc Thesis, Acharya N.G. Ranga Agricultural University.

Sanju, Kamble, K. R.; Pole, S. P.; Bhikane M. U. (2018). Genetic divergence analysis in sunflower (*Helianthus annuus* L.) restorer lines. *International Journal of Current Microbiology and Applied Sciences*, *6*, 742-749.

Seyed Mehdi Safavi; Azam Sadat Safavi (2014). Assessment of genetic diversity in sunflower (*Helianthus annuus* L.) genotypes using agro-morphological traits. *Journal of Biodiversity and Environment Sciences, 6*(1), 152-159.

Sheshaiah; Shankergoud. (2015). Genetic variability and correlation studies in sunflower (*Helianthus annuus* L.). *Electronic Journal of Plant Breeding, 6*(2), 644- 650.

Sowmya, H. C.; Shadakshari, Y.G.; Pranesh, K. J.; Srivastava, A.; Nandini, B. (2010). Character association and path analysis in sunflower (*Helianthus annuus* L.). *Electronic Journal of Plant Breeding, 1*(4), 828-831.

Sudrik, B.P.; Ghodke, M. K.; Patil, V.S.; Chavan, S.K.; Kesale, N.B. (2014). Evaluation and characterization of sunflower (*Helianthus annuus* L.) germplasm. *Journal of Crop and Weed, 10*(1), 73-76.

Supriya, S. M.; Kulkarni, V. V.; Goud, I. S.; Lokesha, R.; Govindappa, M. R. (2016). Genetic variability studies for yield and yield components in sunflower (*Helianthus annuus* L.). *Electronic Journal of Plant Breeding,* ***7***(3), 737-741. https://doi.org/10.5958/0975-928X.2016.00096.X

Supriya, S. M.; Kulkarni, V. V.; Ranganatha, C.N.; Suresha, P.G. (2017). Quantitative analysis of oil yield and its Components in newly developed hybrids of sunflower (*Helianthus annuus* L.). *International Journal of Current Microbiology and Applied Sciences, 6*(8), 3088-3098. https://doi.org/10.20546/ijcmas.2017.608.370

Talele, G. J. (2011*). Effect of supplementary pollination on seed setting and seed quality of sunflower hybrid-Raviraj*. M.Sc (Seed Science and Technology) Thesis, Mahatma Phule Krishi Vidyapeeth.

Tyagi, S. D.; Khan, M. H. (2013). Correlation and path coefficient analysis for seed yield in sunflower (*Helianthus annuus* L.*). International Journal of Agricultural Research, Sustainability, and Food sufficiency*, *1*(2), 07-13.

Tyagi, V.; Dhillon, S. K.; Bajaj, R.K.; Kaur, J. (2013). Divergence and association studies in sunflower (*Helianthus annuus* L.). *Helia, 36*(58), 77-94. https://doi.org/10.2298/HEL1358077T

Venkanna, V.; Reddy, D. L.; Rao, V. T.; Sathish Chandra, B.; Lingaiah, N. (2014). Character association and path analysis for yield and yield attributes in sunflower (*Helianthus annuus* L.). *International Journal of Applied Biology and Pharmaceutical technology, 5*(4), 27-30.

Weber, C.R.; Moorthy, B.R. (1952). Heritable and non-heritable relationship and variability of oil content and agronomic traits in the F2 generations of soybean crosses. *Agronomy Journal, 44*, 202-209. https://doi.org/10.2134/agronj1952.00021962004400040010x

doi:10.14720/aas.2020.116.1.1563 Original research article / izvirni znanstveni članek

Effect of UV-C radiation on basic indices of growth process of winter wheat (*Triticum aestivum* L.) seeds in pre-sowing treatment

Anatoly SEMENOV [1], Irina KOROTKOVA[2, 3], Tamara SAKHNO [1, 2], Mykola MARENYCH [4], Volodymyr HANHUR [5], Viktor LIASHENKO [5], Viktor KAMINSKY [6]

Received March 19, 2020; accepted July 03, 2020.
Delo je prispelo 19. marca 2020, sprejeto 03. julija 2020

Effect of UV-C radiation on basic indices of growth process of winter wheat (*Triticum aestivum* L.) seeds in pre-sowing treatment

Abstract: An investigation of the effects of UV-C radiation from source having the power of 20 W on improvement of seed quality of Ukrainian winter wheat (*Triticum aestivum* L.) six cultivars: 'Gratsiia Myronivska', 'Trudivnytsia Myronivska', 'Vezha Myronivska', 'Mudrist Odeska', 'Nyva Odeska' and 'Lira Odeska', has been done. In the performed experiment the UV-C irradiation in the range of 50-1000 J m^{-2} was applied. Based on the experimental results it is concluded that irradiation of UV-C on wheat seeds stimulated the seed vigour and germination. It has been established the optimal irradiation dose of UV-C radiation - 250 J m^{-2} for 'Trudivnitsia Myronivska', 'Mudrist Odeska', 'Nyva Odeska', 'Lira Odeska' cultivars and 500 J m^{-2} for 'Hratsiia Myronivska' and 'Vezha Myronivska' cultivars, which leads to maximum effect. The highest biometric indices of seedling structural elements were obtained from the seeds of all wheat cultivars, for which the values of seed vigour and germination were also maximum ones, i.e. at the same irradiation dose.

Key words: seed vigour; germination; UV-C radiation; biometric indices; exposure dose

Učinki predsetvenega UV-C obsevanja semen ozimne pšenice (*Triticum aestivum* L.) na osnovne indekse rasti

Izvleček: V raziskavi so bili preučevani učinki UV-C obsevanja, moči 20 W, na izboljšanje kakovosti semen sedmih sort ukrajinske ozimne pšenice *Triticum aestivum*'Gratsiia Myronivska', 'Trudivnytsia Myronivska', 'Vezha Myronivska', 'Mudrist Odeska', 'Nyva Odeska' and 'Lira Odeska'. V poskusu je bilo uporabljeno UV-C sevanje jakosti od 50 do 1000 J m^{-2}. Rezultati so pokazali, da je UV-C sevanje pospešilo vigor pšeničnih semen in njihovo kalitev. Optimalne doze UV-C sevanja so bile za 'Trudivnitsia Myronivska', 'Mudrist Odeska', 'Nyva Odeska', 'Lira Odeska' 250 J m^{-2} in 500 J m^{-2} za 'Hratsiia Myronivska' in 'Vezha Myronivska'. Največje vrednosti biometričnih indeksov strukturnih elementov sejank so bile za vse sorte pšenice dosežene pri isti dozi obsevanja kot maksimalne vrednosti vigorja semen in kalitve

Ključne besede: vigor semen; kalitev; UV-C sevanje; biometrični indeksi; izpostavitvena doza

---

[1] Poltava University of Economics and Trade, Faculty of Commodity science, Trade and Marketing, Department of Commodity research, Biotechnology, Expertise and Customs, Poltava, Ukraine
[2] Poltava State Agrarian Academy, Faculty of Agro-Technology and Ecology, Department of Biotechnology and Chemistry, Poltava, Ukraine
[3] Corresponding author, e-mail: 2irinakorotkova10@gmail.com
[4] Poltava State Agrarian Academy, Faculty of Agro-Technology and Ecology, Department of Selection, Seed Growing and Genetics, Poltava, Ukraine
[5] Poltava State Agrarian Academy, Faculty of Agro-Technology and Ecology, Department of Plant, Poltava, Ukraine
[6] NSC "Institute of Agriculture" NAAS of Ukraine, Kyiv region, Ukraine

1 INTRODUCTION

In the context of seed technology, using physical methods to increase crop productivity has significant advantages in comparison with traditional methods of seed treatment based on chemical compounds (Araújo et al., 2016). Anthropogenic changes of soil, water, and atmosphere as a result of using various chemical additives to increase plant capacity causes searching of the alternative safe methods to increase yields. Such methods envisage the reducing of application of chemical substances or their replacement by physical methods. Among the physical methods used for pre-sowing seed treatment, the following ones are studied most of all: magnetic field, gamma rays, and UV radiation (Govindaraj et al., 2017; Surjadinata et al., 2017). The effectiveness of pre-sowing seeds treatment with electroozonation was proved (Normov et al., 2019).

The results of numerous studies confirm the possibility of magnetic field application for pre-sowing treatment and improving the quality of cereal seeds and in the bean (Balakhnina et al., 2015; Mroczek-Zdyrska et al., 2016; Lazim & Nasur, 2017). There are many hypotheses explaining the action of magnetic fields on seeds and plants, but they are selective. Therefore, the studies to determine the possibility of applying an alternating magnetic field as a factor affecting seed material quality improvement are being continued at present (Pietruszewski & Kania, 2010; Rochalska et al., 2011).

The biological effect of gamma radiation on the main components of wheat cells was studied in Ahmed et al. (2017) and Nazarenko & Izhboldin (2017). It has been established that treatment with gamma radiation leads to changes in the cell structure and cell membrane, photosynthesis, causes the modulation of anti-oxidative system and contributes to the accumulation of phenolic compounds. This effect depends on the radiation dose.

During the previous two decades, a considerable number of experimental papers have been devoted to the study of biological effects and mechanisms of UV radiation influence on plants (Semenov et al., 2017; Kondrateva et al., 2019; Pournavab et al., 2019). There are three potential targets for UV radiation in plant cells: genetic system, photosynthetic system, and membrane lipids. These changes ultimately lead to decreased biomass production and grain yield. The studies of ultraviolet radiation, its doses and other environmental conditions' effect on photosynthesis are of greatest interest (Wenke & Qichang, 2012; Sugimoto, 2013).

It should be noted that the effect of UV radiation on the biology of seeds and their germination is not well understood (Rupiasih & Vidyasagar, 2016). It is known, that seeds react to UV radiation, both as on wave length present in the sunlight (UV-A and UV-B), and wave length below 280 nm (UV-C). But, at present, there is still not enough information about the possible application of UV-C radiation as a stimulating seed treatment.

The stimulating effect of UV-A radiation on legume (*Vigna radiata* L.) seeds is described in (Hamid & Jawaid, 2011). It is mentioned here that pre-treatment of seed with UV-A (366 nm) radiation improves the germination rate, specific leaf area, dry mass, length of roots and shoots as compare to UV-C (254 nm) exposed seed. It is one of the few studies on positive effect of UV-A radiation.

Ultraviolet-B (UV-B) radiation has long been perceived as a stressor. Oxidative stress is flagged as a key factor in such UV-B exposure. Measurements of antioxidants and antioxidant genes show that both low and high doses of UV-B radiation change reactive oxygen species (ROS) metabolism. The elevated level of UV-B radiation can cause a deterioration of seed quality, in particular, contribute to a decrease in protein content, and the total amount of free amino acids (Choudhary & Agrawal, 2014). The authors connect the reduction of protein content with ROS generation, which causes protein oxidative damage. Another reason of seed quality degradation can be the modification and destruction of amino acids under the influence of UV radiation (Peykarestan & Seify, 2012). The obtained results of growth parameters were inversely related to the irradiation doses.

Despite the damaging potential of UV-B photons, which limits photosynthesis and plant growth (Hideg et al., 2013; Choudhary & Agrawal, 2014) it becomes more evident that UV damage is probably the exception than the rule. Thus, the damaging effect of UV-B radiation has now been considered rare. The positive effects of UV-B radiation cover such important areas as stimulation of secondary metabolism, natural means of plant protection, phyto-reagent production and many others (Ballaré et al., 2011). However, as a rule, UV-B radiation is effective only when it lasts for a sufficiently long period of time, usually for several hours or days.

Numerous studies indicate the positive influence of UV-C on seed health, germination and seedling strength of various crops, and also on physiological and biochemical processes in seeds and plants depends on radiation dose (Ouhibi, 2014; Badridze et al., 2016; Castronuovo et al., 2017; Semenov et al., 2018a; Semenov et al., 2018b; Gandhi et al., 2019; Sadeghianfar et al., 2019). But, despite extensive experimental material, fundamental and applied researches are still necessary to determine the optimal irradiation dose, exposure time, depending on plant cultivars and the environment (temperature, humidity).

The aim of this study is to examine the effects of the pre-sowing seed treatment of winter wheat (*Triticum aestivum* L.) with different doses of UV-C radiation (from 50 to 1000 J m^{-2}) on the basic parameters of seed quality: seed vigour and germination as well as growth parameters like length of seedlings shoots and roots.

2 MATERIALS AND METHODS

Three winter wheat (*Triticum aestivum* L.) cultivars from 2018 harvest, 'Gratsiia Myronivska' (1), 'Trudivnitsia Myronivska' (2) and 'Vezha Myronivska' (3), were supplied by the V. M. Remeslo Myronivka Institute of wheat National Academy of Agrarian Sciences of Ukraine. Three winter wheat cultivars from 2018 harvest, 'Mudrist Odeska' (4), 'Nyva Odeska' (5) and 'Lira Odeska' (6), were supplied by the Plant Breeding and Genetics Institute - National Center of Seed and Cultivar Investigation, National Academy of Agrarian Sciences of Ukraine. Experimental studies were carried out in Grain Quality Laboratory of Poltava State Agrarian Academy during 2019.

The seed material was split into two groups: the control and experimental (UV-C). Control seeds were not irradiated. The sample of each cultivar consisted of 100 seeds. The UV lamp of ZW20D15W type (Semenov et al., 2015), 20 W power was used as irradiation source. The intensity of energy irradiation at a distance of 1 m reached 62–69 W cm^{-2}. The measurements of UV-C radiation dose were conducted with the radiometer "Tensor-31" in the range of 200-280 nm (Tensor LLC, Ukraine) using the technique (Semenov et al., 2019).

The seeds of each winter wheat cultivar (100 seeds in quadruple repetitions), including control samples, were placed in a plastic tray on filter paper moistened with distilled water and kept at a temperature of 7 ± 2 °C for 1 day. Seeds were placed in tray at a distance of 0.5 cm from each other. Then the cooled samples (except the control ones) were irradiated with UV-C doses of 50-1000 J m^{-2} with the step of 50 J m^{-2}. The seeds in a single layer were UV-C irradiated on a grid with a cell diameter of 2 mm. The distance between lamps and samples was 25 cm. Irradiation was carried out simultaneously from above and below. By varying of exposure time and distance to the UV source the necessary radiation dose was created.

The seed samples irradiated with different UV-C radiation doses and the control samples were germinated at air temperature of 24 ± 2 °C with the light period of 12/12 h for 14 days.

The seed vigour was determined on day 4, and the germination (the number of seeds that gave normal seedlings in % of 100 seeds) was determined on day 8. As the normal seedlings were considered seedlings with root equal to or more than the length of a seed, and with shoot equal to half of seed length (ISTA, 2017). Further on, the indices of seeds irradiated with different UV-C radiation doses were compared with the non-irradiated control samples. After 14 days of the experiment biometric measurement were conducted. The basic growth parameters such as the average root and seedling lengths were measured with 0.1 cm precision. Biometric measurements were performed in quadruple repetitions for selected 30 seeds at the same physiological age.

The data obtained from the germination and seed vigor laboratory tests were analyzed with the Statistica 10.0 software, where a completely randomized scheme was applied with a bi-factorial arrangement: Factor 1 consisted of the six wheat seeds cultivars, and Factor 2 consisted of the five radiation doses with triplicate repetitions. The statistical data processing was carried out to determine whether there was a significant difference between the means of the treatments. Experimental data was also statistically analyzed for the analysis of variation (ANOVA) and least significant difference (LSD).

3 RESULTS AND DISCUSSION

The reaction of six winter wheat cultivars to different doses of UV-C radiation, showed the stimulating effect of this method of seed treatment. The obtained results of seed vigour and germination are presented graphically in Figures 1 and 2.

As can be seen from Figure 1, seed vigour of 'Gratsiia Myronivska' and 'Vezha Myronivska' cultivars, as a result of 500 J m^{-2} exposure dose, increased by 37 % and 31 %, respectively, compared to the control samples. The UV-C irradiation wheat seeds by a higher dose of 1000 J m^{-2} resulted in decrease of seed vigour to 21-25 % as compared with the seed vigour at irradiation dose of 500 J m^{-2} and to 17-18 % at irradiation dose of 250 J m^{-2}. It should be noted that at seed treatment of 'Gratsiia Myronivska' and 'Vezha Myronivska' cultivars with a dose of 1000 J m^{-2} the number of germinated samples remains at the control level (70 %) taking into account measurement accuracy. This fact indicates a decrease in effectiveness of high radiation doses impact on the seed vigor of these cultivars in comparison with lower radiation doses.

The highest value of seed vigour in 'Trudivnitsia Myronivska', 'Mudrist Odeska', 'Nyva Odeska', 'Lira Odeska' cultivars was registered at a dose 250 J m^{-2}. Comparing seed vigour after treatment relative to the control one can notice an improvement of about 8 % for 'Trudivnitsia Myronivska' cultivar, and of about 30-37 % for 'Mudrist Odeska', 'Nyva Odeska' and 'Lira Odeska' cultivars. Increasing of the irradiation dose to 500 J m^{-2} resulted in

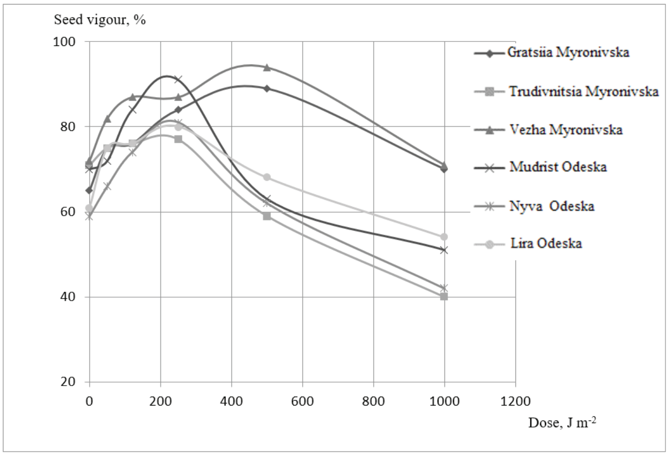


Figure 1: The effect of different dose of UV-C treatment on seed vigour of winter wheat seeds

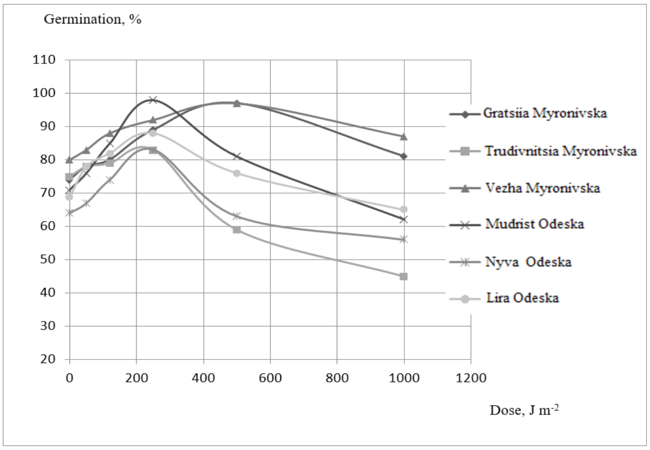


Figure 2: Effect of pre-sowing wheat seed treatment with different dose of UV-C radiation on germination

decrease of seed vigour to 15-31 % as compared with the control. As a result of exposure to a higher dose of 1000 J m^{-2}, the seed vigour of 'Trudivnitsia Myronivska', 'Mudrist Odeska' and 'Nyva Odeska' cultivars decreased compared with control by 27-44 % and of 'Lira Odeska' cultivar by 12 %.

Thus, the obtained results confirm that applying UV-C irradiation with doses of 500-1000 J m^{-2} for pre-sowing seed treatment of 'Gratsiia Myronivska' and 'Vezha Myronivska' cultivars, and a dose of 1000 J m^{-2} for 'Trudivnitsia Myronivska', 'Mudrist Odeska', 'Nyva Odeska' and 'Lira Odeska' cultivars will definitely have worsened effect on the sowing qualities of the studied wheat cultivars' seeds.

The magnitude of seed vigour correlates well with germination, which is of interest for present research. The stimulating effect of UV irradiation was found for all wheat seed cultivars including those with low germination in control: for 'Nyva Odeska' and 'Lira Odeska' cultivars the control germination amounted of about 64 % and 69 %, respectively (Figure 1, 2).

As a result of UV treatment with a dose 250 J m^{-2}, germination of 'Nyva Odeska' cultivar increased by 30 % and amounted to 83.3 %, and germination of 'Lira Odeska' cultivar increased by 27 % and amounted to 88 %. It should be noted that the germination of 'Nyva Odeska' cultivar after treatment of 500 J m^{-2} practically did not change as compared with the control and amounted 63 %. The maximum germination of 98 % among all tested cultivars was registered for seeds of 'Mudrist Odeska' cultivar for irradiation dose of 250 J m^{-2}. The irradiation of seeds this cultivar by a dose 250 J m^{-2} led to an increase in germination on 38 % as compared to control seeds.

Weak effect of UV radiation was observed for seeds of 'Trudivnitsia Myronivska' cultivar at irradiation doses of a smaller range from 50 to 200 J m^{-2}. Only as a result of seed treating by dose of 250 J m^{-2} the germination increase by 10.7 % was observed.

The seeds of 'Gratsiia Myronivska' and 'Vezha Myronivska' cultivars turned out to be the most sensitive to UV-C of 500 J m^{-2} exposure doses as in determining seed vigour. Germination of these cultivars after treatment increased by 31.1 % and 21.1 %, respectively, compared with the control.

With increasing radiation dose to 1000 J m^{-2} there was a decrease of germination by 6-13 % for 'Mudrist Odeska', 'Nyva Odeska' and 'Lira Odeska' cultivars (Figure 2). At such radiation dose, the lowest germination of 'Trudivnitsia Myronivska' cultivar seeds (about 45 %) was registered, which on 40 % lower the germination of the control sample (75 %). This is the lowest index of germination for all the tested cultivars obtained as a result UV-C radiation treatment.

It can be determined from the established dependence of seed vigor and germination on UV-C radiation dose (Figure 1, 2) that the optimal dose for pre-sowing seed treatment of the 'Trudivnitsia Myronivska', 'Mudrist Odeska', 'Nyva Odeska', 'Lira Odeska' cultivars is 250 J m^{-2} and 500 J m^{-2} for 'Hratsiia Myronivska' and 'Vezha Myronivska' cultivars. As a result of seed treated with optimal doses the seed vigor and germination are the highest. Our results confirm the data from the study by Tertyshnaya et al. (2018) on examining the impact of UV-B radiation (365 nm) on seed germination of spring and winter wheat. The authors have shown that wheat germination 'Moskovskaya 39' (67 %), 'Yubileynaya 100' (92 %), 'Dar'ya' (98 %) and 'Afina' (99 %) cultivars increased by 1-3 % as a result of seed treatment. Insignificant germination increases as compared to our results can be explained by the difference in wavelength of UV-B and UV-C ranges, and as a consequence, different irradiation efficiencies.

A significant increase in germination by 38 % at the established optimal radiation dose of 250 J m^{-2} obtained in present study (the investigated range is 50-1000 J m^{-2}) is in consistent with results observed in Rupiasih & Vidyasagar (2016), where the influence of different period of exposure (30, 60, 90, 120 and 180 minutes) using a UV-C source from Pride (240 V, 50 Hz; 20 W) on the effectiveness of wheat seeds germination has been studied. The results showed that there is no direct dependence between the exposure periods and seed germination. Maximum germination of 96.7 % was recorded at 180 min UV-C irradiation treatment, and it was low (83.3 %) at 60 min UV-C treatment that is equal to control. At the same time, the minimum germination of 67 % was recorded at 120 min treatment (Rupiasih & Vidyasagar, 2016).

The similar dependence was also observed for seeds of other crops, as shown in study by Siddiqui et al. (2011). An experiment was conducted on seeds of mung bean (*Vigna radiata* (L.) Wilczek and groundnut (*Arachis hypogaea* L.) exposed to ultraviolet (UV-C < 280 nm) radiations for 0, 5, 10, 15, 20, 30 and 60 minutes' period. As a result, the optimal exposure time leading to the seed stimulation of these crops was determined. UV-C exposure seed for 30 min period gave significant result in increment of germination of mung bean (up to 100 %) and groundnut (up to 86.66 %).

According to the results of multi-factor analysis of variance (Factorial ANOVA) it has been revealed that cultivars properties play an important role in genotype reaction on irradiation factor in determining seed vigor (LSD_{05} = 2.0%). The value of least significant difference (LSD_{05}) was 4.5 % for factor interaction and 5.0 % for the index of seed laboratory germination.

Thus, it was found that 28 % of the total seed vigor variation is explained by cultivar properties, and 67 % due to the radiation dose. For germination indices, these values were almost the same, namely 46 % of the total germination variation was stipulated by cultivar characteristics, and 47 % – by radiation dose.

The analysis of variance (Table 1) showed that wheat cultivar characteristics play a much smaller role for seed vigor as compared with germination.

The value of F criterion in calculating seed vigor for the cultivar factor and for the radiation dose factor was 117.38 and 285.36 respectively. The value of germination depended on cultivar characteristics and irradiation dose

Table 1: Result of the variance analysis to determine the seed vigour and germination

	Univariate tests of significance for seed vigour/germination, %				
Effect	SS	Degree of freedom	MS	F	*p*
Intercept	551265,3 / 646197,4	1	551265,3 / 646197,4	76920,74/76023,22	0,00
Cultivar	4206,0 / 5308,3	5	841,2 / 1061,7	117,38 / 124,90	0,00
Dose, J m^{-2}	10225,4 / 5516,1	5	2045,1 / 1103,2	285,36 / 129,79	0,00
Cultivar* Dose, J m^{-2}	4081,2 / 4014,3	25	163,2 / 160,6	22,78 / 18,89	0,00
Error	516,0 / 612,0	72	7,2 / 8,5		

Note: The table shows the main results of the analysis: Sum-of-Squares (SS), Mean Squares (MS), Degree of freedom, value of F criterion (F), significance levels (p).

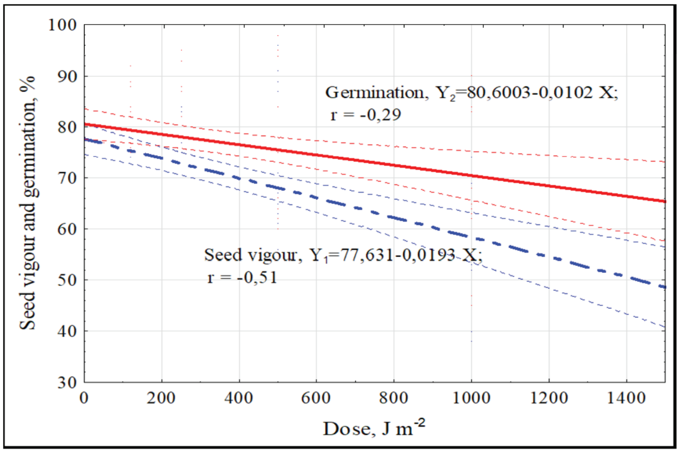


Figure 3: Seed vigour and germination regression plot from UV-C radiation dose

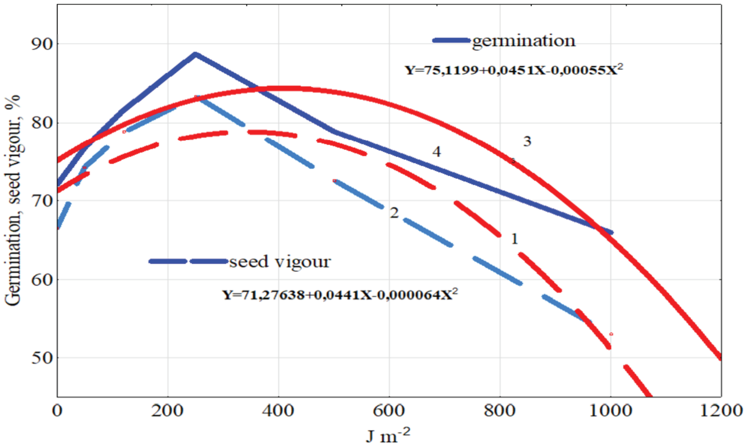


Figure 4: Experimental and theoretical curves dependence of seed vigour (1, 2) and germination (3, 4) of wheat seeds from dose UV-C treatment (1, 3 - theoretical curves; 2, 4 - experimental curves)

almost equally – the interaction of factors at the effect on both indicators was F = 124.90 and 129.79 at $p < 0.01$.

It was also established that with an increase in radiation dose, the correlation value between the seed vigor and germination increase from r = 0.74 for the control samples to r = 0.88 at radiation dose of 500 J m^{-2}. Later this correlation weakened. Thus, it was statistically founded there is an average and weak inverse correlation between the indicators of seed vigour and germination and radiation doses.

The regression equation is presented in Figure 3. The presence of such conformities indicates the probability of the existence of another type of dependence. The analysis of the experimental results by non-linear regression method showed that for the irradiation factor, the graphical dependence has the parabola shape. Experimental curves and theoretical equations of functions' dependence of seed vigor and germination on the radiation dose are given in Figure 4. The theoretical curves were selected in such a way that they reflected the obtained experimental dependence. In our case, the equations of the quadratic function are best superimposed on the experimental curves (parabolas of the form $Y = b_0 + b_1X - b_2X^2$). The regression coefficients in this case are valid at $p < 0.01$.

It was experimentally established that the highest values of seed vigor and germination for 'Trudivnitsia Myronivska', 'Mudrist Odeska', 'Nyva Odeska' and 'Lira Odeska' wheat cultivars were obtained at radiation dose of 250 J m^{-2} and for 'Gratsiia Myronivska' and 'Vezha Myronivska' cultivars – at radiation dose of 500 J m^{-2}. However, theoretical calculations show that the improvement in seed vigor and germination indices can be achieved under applying 400-500 J m^{-2} doses.

Two weeks after sprouting, biometric evaluation of seedling structural elements was conducted. For this

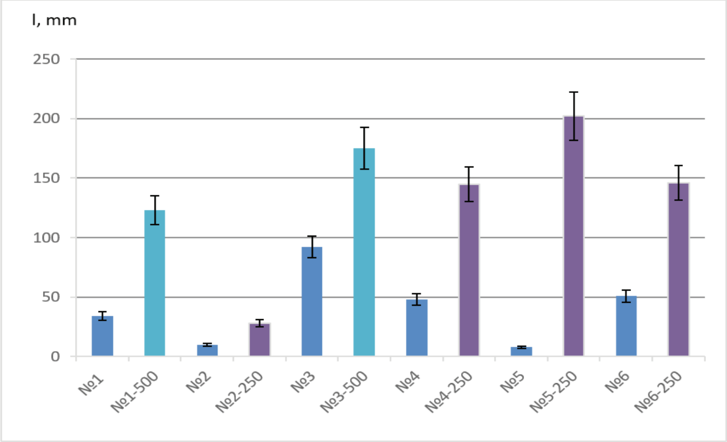


Figure 5: Effect of UV-C radiation on root length of wheat seeds at different exposure dose applied (LSD_{01} = 2.00 mm)

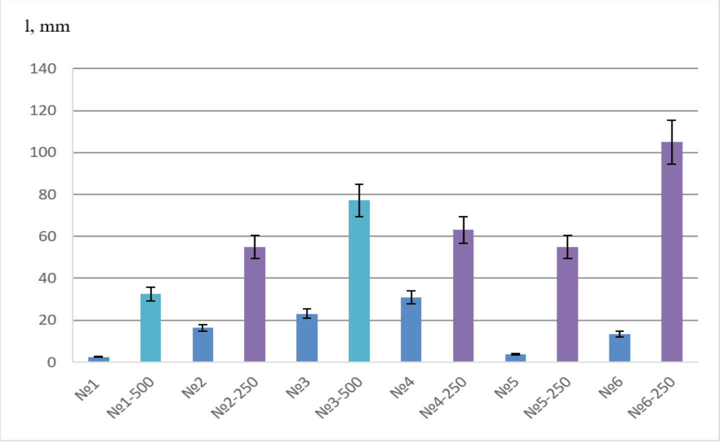


Figure 6: Effect of UV-C radiation on shoot length of wheat seeds at different exposure dose applied (LSD_{01} = 2.2 mm)

purpose, 30 seedlings in quadruple repetitions were selected carefully (without breaking the root system) from each of the trays and length of roots and the aboveground parts of plants were measured. The result of UV-C radiation stimulating effect was the improvement of biometric indices in all the investigated cultivar seeds. The height of seedlings and length of root system depending on the dose and cultivar were not the same.

Significant effect of UV irradiation on development of the root system and aboveground part was observed in seeds treated with 250 J m^{-2} and 500 J m^{-2} doses of UV-C radiation**,** at which seed vigor and germination had maximum values. Length of root system in all wheat cultivars increased by 2.0–3.6 times on the average, and length of shoots increased by 2.0-3.4 times as a result of irradiation (Figure 5, 6).

The maximum effect of UV-C radiation on root length and height of shoots was observed in seeds of ‘Nyva Odeska’ cultivar. So, the length of seedlings roots exposed with 250 J m^{-2} dose 25 times exceeded length of roots of control samples (Figure 5, №5) and reached 202 mm (Figure 5, №5-250), and the height of shoots increased by 14.4 times and amounted to 55 mm (control samples – 3.8 mm) as shown at Figure 6. The obtained results correlate well with seed vigour and germination at dose of 250 J m^{-2}. The values of seed vigour and germination after treatment compared with the control were founded higher of about 37 % and 30 %, respectively. The effect of UV-C irradiation (200-280 nm) on biometric parameters of the wheat cultivars studied by us turned out to be more significant than that described in study Tertyshnaya et al. (2018). As it was established in our experiments, the length of seedlings roots of all the investigated seed cultivars increased by 2-3.6 times, and height of shoots – by 2.0-3.4 times, while the authors of study Tertyshnaya et al. (2018) observed increasing the root length of wheat seedlings treated with UV-B rays (365 nm) by only 26-60 %. The height of shoots obtained from seeds treated with UV-B for 30 minutes increased as compared to control on 30 % in 'Moskovskaya 39' cultivar, in 'Yubileynaya 100', 'Dar'ya' and 'Afina' cultivars – by only 3-5 %. Insignificant increases of the seedlings root length and shoot height as compared to our results can be explained by the difference irradiation efficiencies of the UV-B and UV-C ranges.

The effectiveness of UV-C ray pre-sowing treatment is shown on the example of mung bean and peanut seeds (Siddiqui et al., 2011), but the optimum exposure time affecting the root length of and seedling height was not determined by the authors. So, maximum shoot height of 22.38 ***cm*** compared with the control (13 cm) was observed when seeds of mung bean were treated with UV-C for 15 minutes while root length was increased to 19.58 cm (control - 10.53 cm) when seeds were treated for 30 minutes. The increase in shoot length of groundnut seeds (6.83 cm vs. 4.91 cm in the control) was attained when seeds were treated with UV-C radiation for 60 minutes whereas maximum root length was observed in Siddiqui et al. (2011) when seeds were treated with UV-C for 30 minutes (18.39 cm vs. 13.62 cm in the control).

Dose-dependent reaction of lettuce seeds treated with 0.82 kJ m^{-2} and 3.42 kJ m^{-2} of UV-C radiation dose was established Ouhibi et al. (2014). Seed irradiation with the lowest dose of 0.85 kJ m^{-2} contributed to increasing length of roots and pre-serving dry mass under salinity stress.

However, there are experimental studies indicated the negative effect of UV-C (254 nm) radiation at different period of exposure for 30 and 60 min on seeds of various crops compared to untreated seeds (Lazim & Nasur, 2017). So, at 60 min exposure time the lowest seedling length (11.09 cm) which reduced by (24.04 %) compared to control (14.6 cm) was observed. Significant effect in root length of sorghum seeds between all treatments periods was not found.

Thus, the obtained results enable to conclude that for different cultivar seeds there is an optimum amount of absorbed radiation energy leading to the maximum effect. These could be attributed to the difference in the structure of the seeds, physiological state, composition, and epidermis thickness of the seeds coat.

4 CONCLUSIONS

The present study shows that UV-C radiation may have a significant effect on the sowing properties of the tested winter wheat cultivars of Ukrainian selection. The optimal doses of UV-C radiation - 250 J m^{-2} for 'Trudivnitsia Myronivska', 'Mudrist Odeska', 'Nyva Odeska', 'Lira Odeska' cultivars and 500 J m^{-2} for 'Hratsiia Myronivska' and 'Vezha Myronivska' cultivars were established. Seed treatment with optimal radiation doses of all wheat cultivars contributed to the increase in seed vigour by 30-37 %, and germination by 21-38 % as compared to control samples.

It is shown that UV irradiation positively affects the biometric parameters of wheat seedlings. The root system of irradiated seed samples is more developed than of unexposed ones. As a result of UV-C radiation with established doses, the root length in all the studied wheat cultivars significantly increased by 2.0-3.6 times, and the seedling shoot height - by 2.0-3.4 times. The maximum increase in root length by 25 times and shoot height – by 14.4 times was found in seed seedlings of 'Nyva Odeska'.

The obtained results are of interest and prove the expediency of using UV-C irradiation to increase the sowing qualities of large batches of seeds with reduced germination. The proposed method of stimulating seeds with UV-C radiation can be applied in selection, where sometimes there is a threat of losing small amounts of valuable breeding material with low seed vigour and germination.

5 REFERENCES

Araújo, S. de S., Paparella, S., Dondi, D., Bentivoglio, A., Carbonera, D., Balestrazzi, A. (2016). Physical Methods for Seed Invigoration: Advantages and Challenges in Seed Technology. *Frontiers in Plant Science, 7*, 646. https://doi.org/10.3389/fpls.2016.00646

Ahmed, S., Khan, W.M., Khan, M.S., Akhtar, N., Umar, N., Ali, S., Hussain, S., & Shah, S.S. (2017). Impact of gamma radiations on wheat (*Triticum aestivum* L.) varieties (Batoor and Janbaz). *Pure* and *Applied Biology, 6*(1), 218–225. https://doi.org/10.19045/bspab.2017.60017

Balakhnina, T., Bulak, P., Nosalewicz, M., Pietruszewski, S., Włodarczyk, T. (2015). The influence of wheat *Triticum aestivum* L. seed pre-sowing treatment with magnetic fields on germination, seedling growth, and antioxidant potential under optimal soil watering and flooding. *Acta Physiologiae Plantarum, 37*, 59. https://doi.org/10.1007/s11738-015-1802-2

Ballaré, C.L., Caldwell, M.M., Flint, S.D., Robinson, S.A., Bornman, J. F. (2011). Effects of solar ultraviolet radiation on terrestrial ecosystems. Patterns, mechanisms, and interactions with climate change. *Photochemical & Photobiological Sciences, 10*, 226–241. https://doi.org/10.1039/c0pp90035d.

Badridze, G., Kacharava N., Chkhubianishvili E., Rapava L., Kikvidze M., Chanishvili S., Shakarishvili N., Mazanishvili L., Chigladze, L. (2016). Effect of UV radiation and artificial acid rain on productivity of wheat. *Russian Journal of Ecology, 47*(2), 158-166. https://doi.org/10.1134/S106741361602003X

Castronuovo, D., Sofo A., Lovelli S., Candido V., Scopa A. (2017). Effects of UV-C radiation on common dandelion and purple coneflower: first results. *International Journal of Plant Biology, 8*(1), 7255. https://doi.org/10.4081/pb.2017.7255

Choudhary, K. K., & Agrawal, S.B. (2014). Ultraviolet-B induced changes in morphological, physiological and biochemical parameters of two cultivars of pea (*Pisum sativum* L.). *Ecotoxicology and Environmental Safety, 100*, 178–187. https://doi.org/10.1016/j.ecoenv.2013.10.032

ISTA. (2017). *International rules for seed testing*, 2017 (1): i-5-56(56). International Seed Testing Association. https://doi.org/10.15258/istarules.2017.05

Gandhi, N., Rahul K., Chandana N., Madhuri B., Mahesh D. (2019). Impact of ultraviolet radiation on seed germination, growth and physiological response of Bengal gram (*Cicer arietinum* L.) and horse gram (*Macrotyloma uniflorum* L.). *Journal of Biochemistry Research, 2*(1), 019–0034.

Govindaraj, M., Masilamani, P., Albert, A.V., & Bhaskaran M. (2017). Effect of physical seed treatment on yield and quality of crops: A review. *Agricultural Reviews, 38*(1), 1-14. https://doi.org/10.18805/ag.v0iOF.7304

Hamid, N., Jawaid, F. (2011). Influence of Seed pre-treatment by UV-A and UV-C radiation on germination and growth of Mung beans. *Pakistan Journal of Chemistry, 1*(4), 164-167. https://doi.org/10.15228/2011.v01.i04.p04

Hideg, E., Jansen, M.A.K., & Strid, A. (2013). UV-B exposure, ROS, and stress: inseparable companions or loosely linked associates? *Trends in Plant Science, 18***,** 107–115. https://doi.org/10.1016/j.tplants.2012.09.003

Kondrateva, N.P., Krasnolutskaya M.G., Dukhtanova N.V., Obolensky N.V. (2019). Effect of ultraviolet radiation the germination rate of tree seeds. *IOP Conf. Series: Earth and Environmental Science, 226: 012049*. https://doi.org/10.1088/1755-1315/226/1/012049

Lazim, S.K., & Nasur A.F. (2017). The effect of magnetic field and ultraviolet-C radiation on germination and growth seedling of sorghum (*Sorghum bicolor L. Moench*). *Journal of Agriculture and Veterinary Science, 10*(10), 30-36. https://doi.org/ 10.9790/2380-1010023036

Mroczek-Zdyrska, M., Tryniecki L., Kornarzyński K., Pietruszewski S., Gagoś M. (2016). Influence of magnetic field stimulation on the growth and biochemical parameters in *Phaseolus vulgaris* L. *Journal Microbiology, Biotechnology and Food Science, 5*(6), 548-551. https://doi.org/10.15414/jmbfs.2016.5.6.548-551

Nazarenko, M.M., Izhboldin, O.O. (2017). Chromosomal rearrangements caused by gamma-irradiation in winter wheat cells. *Biosystems Diversity, 25*(1), 25-28. https://doi.org/10.15421/011704

Normov, D., Chesniuk, E., Shevchenko, A., Normova, T., Goldman, R., Pozhidaev, D., Bohinc, T., Trdan S. (2019). Does ozone treatment of maize seeds influence their germination and growth energy? *Acta agriculturae Slovenica*, *114/2*, 251–258. https://doi:10.14720/aas.2019.114.2.10

Ouhibi, C., Attia, H., Rebah, F., Msilini, N., Chebbi, M., Aarrouf, J., Urban, L., Lachaal, M. (2014). Salt stress mitigation by seed priming with UV-C in lettuce plants: Growth, antioxidant activity and phenolic compounds. *Plant Physiology and Biochemistry, 83***,** 126–133. https://doi.org/10.1016/j.plaphy.2014.07.019

Pietruszewski, S., Kania, K. (2010). Effect of magnetic field on germination and yield of wheat. *International Agrophysics, 24***,** 297-302.

Peykarestan, B., Seify, M.R. (2012). UV Irradiation Effects on Seed Germination and Growth, Protein Content, Peroxidase and Protease Activity in Red Bean. *International Journal of Science and Engineering Investigations, 1*(3), 107–113.

Pournavab, R.F., Mejía E.B., Mendoza A.B., Cruz L.R.S., Heya M.N. (2019). Ultraviolet radiation effect on seed germination and seedling growth of common species from Northeastern Mexico. *Agronomy, 9* (6), 269. https://doi.org/10.3390/agronomy9060269

Rochalska, M., Grabowska-Topczewska, K., Mackiewicz, A. (2011). Influence of alternating low frequency magnetic field on improvement of seed quality. *International Agrophysics, 25***,** 265-269.

Rupiasih, N.N., & Vidyasagar P.B. (2016). Effect of UV-C radiation and hypergravity on germination, growth and content of chlorophyll of wheat seedlings. *AIP Conference Proceedings, 1719: 030035*. https://doi.org/10.1063/1.4943730

Sadeghianfar, P., Nazari M., & Backes G. (2019). Exposure to Ultraviolet (UV-C) Radiation Increases Germination Rate of Maize (*Zea maize* L.) and Sugar Beet **(***Beta vulgaris***)** Seeds. *Plants, 8*(2), 49. https://doi.org/10.3390/plants8020049

Siddiqui, A., Dawar, S., Zaki, M.J., Hamid N. (2011). Role of Ultra Violet (UV-C) radiation in the control of root infecting fungi on groundnut and mung bean. *Pakistan Journal of Botany, 43*(4), 2221-2224.

Surjadinata, B.B., Jacobo-Velázquez, D.A., Cisneros-Zevallos, L. (2017). UVA, UVB and UVC Light Enhances the Biosynthesis of Phenolic Antioxidants in Fresh-Cut Carrot through a Synergistic Effect with Wounding. *Molecules, 22*, 668–681. https://doi.org/10.3390/molecules22040668.

Sugimoto, K. (2013). Seed germination under UV-B irradiation. *Bulletin Minami-Kyushu University, 43A*, 1–9.

Semenov, A., Kozhushko G., Bala L. (2015). No ozone germicidal lamp for plants photochemical and photo biological action. *Technological audit and production reserves, 4/1*(24), 4–7. https://doi.org/10.15587/2312-8372.2015.46953.

Semenov, A.O., Kozhushko G.M., Sakhno T.V. (2017). Analysis of the role of UV radiation on the development and productivity of different cultures. *Light engineering and electricity, 2*, 3-16. http://nbuv.gov.ua/UJRN/svitteh_2017_2_3

Semenov, A., Kozhushko, G., Sakhno, T. (2018 a). Influence of pre-sowing UV-radiation on the energy of germination capacity and germination ability of rapeseed. *Technology Audit and Production Reserves, 5/1*(43), 61–65. https://doi.org/10.15587/2312-8372.2018.1434

Semenov, A.O., Burhu Yu.G., Kozhushko G.M., Marenych M.M., Sakhno T.V. (2018 b). Influence of ultraviolet radiation on germination, sprouting and growth processes of wheat. *Bulletin of Poltava State Agrarian Academy, 4,* 70–75. https://doi.org/10.31210/visnyk2018.04.10

Semenov, A., Korotkova, I., Sakhno, T., Marenych, N. (2019). The exploiting of agronomic potential of UV-C irradiation for increasing the pre-sowing qualities of the carrot seeds. *Ukrainian Black Sea Region Agrarian Science, 1*(101), 47-52. https://doi.org/10.31521/2313-092X/2019-1(101)-7

Tertyshnaya, Yu.V., Levina, N.S., Elizarova, O.V. (2018). Impact of ultraviolet radiation on germination and growth processes of wheat seeds. *Agricultural Machinery and Technologies, 2*, 31-36. https://doi.org/10.22314/2073-7599-2018-11-2-31-36

Wenke, L., & Qichang, Y. (2012). Effects of day-night supplemental UV-A on growth, photosynthetic pigments and antioxidant system of pea seedlings in glasshouse. *African Journal of Biotechnology, 11*(82), 14786–14791. https://doi.org/10.5897/AJB12.2020

doi:10.14720/aas.2020.116.1.1116 Original research article / izvirni znanstveni članek

Titanium dioxide nanoparticles alleviate cadmium toxicity in lentil (*Lens culinaris* Medic) seeds

Hassan FEIZI [1, 2], Nafiseh AGHELI [1], Hossein SAHABI [1]

Received April 11, 2019; accepted May 29, 2020.
Delo je prispelo 11. aprila 2019, sprejeto 29. maja 2020.

Titanium dioxide nanoparticles alleviate cadmium toxicity in lentil (*Lens culinaris* Medic) seeds

Abstract: This research was carried out with the aim of investigating the effects of different concentrations of titanium dioxide nanoparticles and cadmium on lentil seed germination and seedling early growth. The experiment was conducted as a factorial layout based on a completely randomized design with four replicates. Test plants were exposed to 0, 100, 250, and 500 ppm of cadmium, and then amended with 0, 100, 200, and 300 ppm of TiO_2 nanoparticles. Results demonstrated that the application of 300 ppm of titanium dioxide nanoparticles improved the mean germination time (MGT) and seed germination rate by 39 % and 62 % respectively. Most traits exhibited a decreasing trend as the concentration of cadmium increased. Furthermore, application of 500 ppm of cadmium concentration increased mean germination time compared to the control group (1.667 day). In conclusion, the application of titanium dioxide nanoparticles averted the effect of high cadmium stress on lentils, and maintained the length of the shoot compared to the control. However, further studies on plant life cycles should be performed to detect the inhibitory effects of titanium dioxide on heavy metal toxicity.

Key words: heavy metals; cadmium; germination; nanoparticles

Nanodelci titanovega dioksida zmanjšujejo strupenost kadmija pri kalečih semenih leče (*Lens culinaris* Medic)

Izvleček: Namen raziskave je bil preučiti učinke različnih koncentracij nanodelcev titanovega dioksida in kadmija na kalitev semen leče in zgodnjo rast njenih sejank. Poskus je bil izveden kot popolni naključni faktorski poskus s štirimi ponovitvami. Testirane rastline so bile izpostavljene 0, 100, 250 in 500 ppm kadmija, s kasnejšim dodatkom 0, 100, 200 in 300 ppm nanodelcev TiO_2. Rezultati so pokazali, da je dodatek 300 ppm nanodelcev titanovega dioksida izboljšal poprečni čas kalitve (MGT) za 39 % in hitrost kalitve za 62 %. Večina merjenih znakov je pokazala padajoč trend s povečevanjem koncentracije kadmija. Uporaba 500 ppm koncentracije kadmija je povečala poprečni čas kalitve za 1,667 dni v primerjavi s kontrolo. Zaključimo lahko, da je pri leči uporaba nanodelcev titanovega dioksida odpravila učinke stresa velikih koncentracij kadmija in ohranila dolžino poganjkov primerljivo s kontrolo. Vsekakor pa so potrebne še nadaljne raziskave življenskega kroga rastlin za ugotavljanje inhibitornih učinkov titanovega dioksida na toksičnost težkih kovin.

Ključne besede: težke kovine; kadmij; kalitev; nanodelci

---

[1] University of Torbat Heydarieh, Department of Plant Production, Torbat Heydarieh, Iran
[2] Corresponding author, e-mail: h.feizi@torbath.ac.ir

1. INTRODUCTION

Nowadays, the need for agricultural production, along with the demand for plant raw materials for food production, has increased in the world. In this condition, the use of some branches of the industry in new research and the adoption of safe decisions are necessary to increase agricultural production. Anthropogenic activity degrades the soil, water, and atmosphere and this has led to the search for sustainable ways to improve food production (Feizi & Rezvani Moghadam, 2010).

Soil contamination with heavy metals is one of the most important environmental problems worldwide. Upon gaining entry into agricultural lands, heavy metals can be absorbed by plants and transferred to humans through the food chain. The increase in the heavy metal content in the human body is due to the consumption of plant products grown in areas contaminated with these metals, inhaling soil particles and consuming contaminated water (Motesharezadeh et al., 2014). Among heavy metals, cadmium is one of the most toxic elements which has no biological role in humans. Cadmium (Cd) is an unnecessary element with high biological durability causing tubalization of leaves, chlorosis and reduced root and stem growth (Ahmadvand et al., 2012). In plants, Cd exposure frequently triggers negative effects on their growth and productivity. Recently, it has been proved that Cd can also increase root length and number, and consequently, root area and volume. In plant shoots, Cd can improve the content of photosynthetic pigments and enzymatic and non-enzymatic antioxidants are enhanced in both roots and shoots (Carvalhoa, et al. 2020).

Although plants need some heavy metals such as zinc, iron, nickel, titanium etc. at very low concentrations, however, it leads to metabolic disorders and inhibitory growth of most plant species when the concentration of these metals is higher than the plant requirement. Recently, scientists and researchers have begun to develop efficient and inexpensive technologies, such as the use of microorganisms and living plants to remediate contaminated areas. (Ahmadvand et al., 2012).

Titanium is one of the beneficial elements for plant, and can stimulate the absorption of other elements such as nitrogen, phosphorus, calcium, magnesium, iron, manganese and zinc (Pais, 1983). TiO_2 nanoparticles have shown considerable advantages in absorbing and concentrating chemical pollutants owing to their exclusive properties such as high surface area-to-volume ratio, enhanced surface activity, and high affinity (Deng et al., 2017). In one study on fennel plants, it was observed that treatment with 60 ppm titanium dioxide nanoparticles significantly improved the seed germination percentage and germination rate (Feizi et al., 2013). In addition, the highest mean activity of ascorbate peroxidase, guaiacol peroxidase, and catalase enzymes on annual medic plant (*Medicago scutellata* L.) was found in foliar application of titanium nanoparticles in 0.06 % concentration (Dolatabadi et al., 2015).

Previous studies indicated that TiO_2 nanoparticles had high efficiency and adsorption rate for Pb, Cd, Cu, Zn, and Ni (Engates & Shipley, 2011). The potential influence of TiO_2 nanoparticles on the uptake and toxicity of heavy metals in biological systems has been documented. TiO_2 nanoparticles significantly enhanced the bioaccumulation and toxicity of Pb, Cu, As(III), Zn, and Cd in zebra fish (*Danio rerio* HAMILTON, 1882) larvae, *Daphnia magna* Straus, 1820, and carp (*Cyprinus carpio* Linnaeus, 1758) (Miao et al., 2015; Tan & Wang, 2014). However, Chen et al. (2015) reported that the bioavailability and toxicity of Cd and Cu are reduced in alga (*Chlamydomonas reinhardtii* P.A.Dang) and (*Microcystis aeruginosa* Kützing) and amphipod (*Gammarus fossarum* Linnaeus, 1758) by simultaneous exposure to TiO_2 nanoparticles. Although both stimulatory and inhibitory impacts of nanoparticles on various crop species have been reported in the literature, there is limited information on the impact of TiO_2 nanoparticles in heavy metal stress.

This work investigated the impact of TiO_2 nanoparticles in ameliorating the hazardous effects of Cd on seed germination and seedling growth of lentil. Furthermore, it assesses the co-effect of TiO_2 nanoparticles and cadmium on lentil seedling. Therefore, our objectives were to (i) survey Cd-stressed lentil seed germination parameters using TiO_2 nanoparticles, (ii) evaluate inhibitory effects of cadmium on lentil seedling and (iii) evaluate protective role of TiO_2 nanoparticles under cadmium toxicity conditions.

2 MATERIALS AND METHODS

This research was carried out in the laboratory of Medicinal plants of Faculty of Agriculture, University of Torbat Heydarieh, Iran with the aim of investigating the co-effects of different concentrations of titanium dioxide nanoparticles and cadmium sulfate on lentil seed of Gachsaran cultivar (based on literature minimum concentration of Cd that could harm the lentil is unknown). This experiment was conducted as a factorial based on a completely randomized design with four replicates (in total 1600 seeds). We had a total 16 treatment combinations. We had four replicate for 16 treatments and considered 25 seeds in each treatment (25 seeds into each Petri dish). Consequently we calculated 4 × 4 × 4 × 25 = 1600 seeds. The first factor consisted of different levels of cadmium sulfate concentration of 0, 100, 250 and 500

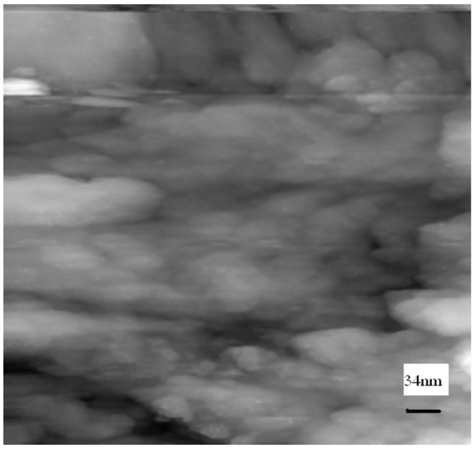


Figure 1: Image of nanosized TiO_2 by Scanning Tunneling Microscope (STM)

ppm, and the second factor included the concentrations of TiO_2 nanoparticles of 0, 100, 200 and 300 ppm. Cadmium sulfate ($CdSO_4$) as a salt of cadmium was used for preparing cadmium stress treatment. First, healthy lentil seeds were selected and disinfected with sodium hypochlorite 5 % for 60 seconds, then washed with distilled water three times.

The titanium dioxide nanoparticle powder was AEROXIDE® TiO_2 P25 prepared from Evonik Degussa GmbH Company of Germany. The purity of nanoparticle powder was 99.8 % and the mean particle size was 21 nm. The required amount for each treatment level of titanium dioxide was weighted using a digital scale with a precision of 0.0001 g and by adding distilled water as the suspension. In order to obtain properly dispersed and stable TiO_2 suspensions of each concentration, an ultra-sonication treatment was applied to nanoparticles TiO_2 powders dispersed in water for 15 min. After preparation, the nanoparticles were poured in different concentrations at separate Erlenmeyer flasks and the seeds were soaked in each concentration for 2 hours. The Erlenmeyer flasks were placed on a shaker to prevent the deposition of nanoparticles, so that the seeds had an identical contact surface with nanoparticles during the treatment. The size of TiO_2 nanoparticles (Figure 1) were determined by Scanning Tunneling Microscope (STM) in Central Laboratory of Ferdowsi University of Mashhad, Iran.

Then, they were placed in a 25-seed group with an appropriate distance from each other in each Petri dish. The experimental unit consisted of a sterile Petri dish with a culture medium of filter paper type (TP or paper culture method). Filter paper was packed in a double layer of aluminum foil for disinfection and placed in oven for 2 hours at a temperature of 120 °C, then, 5 ml of cadmium sulfate solution was added to each Petri dish. Distilled water was used for control treatment. Petri dishes were placed inside the germinator at 23 ± 1 ° C at 16/8 hours length in day / night (ISTA, 2009). The daily count of germinated seeds was performed every 12 hours in each experimental unit and seeds with seminal root length of more than two millimeters were counted as germinated seeds (ISTA, 2009). After a period of time necessary for planting seeds (10 days), ten seeding selected in each Petri dish. The length of seminal root and shoot was measured with the ruler and then, the roots and shoots were separated and placed them individually inside the pocket and placed in an oven at 70 °C for 48 hours. After drying the shoots and roots, they were weighed per treatment individually.

In this experiment, traits such as the length of seminal root, and seedling, seedling mass, germination rate, germination percentage, seed vigor index I and II were evaluated.

On the tenth day, the count of seminal root, shoot and whole seedling was measured and then the seedling dry weight was determined in the oven for a suitable time. Germination rate was calculated by the Maguire (1982) formula as equation (1):

$$\mathit{Germination\ Rate}\ (\mathit{GR}) = (a \backslash 1) + (b - a / 2) + (c - b \backslash 3) + \ldots + (n - n - 1 / N) \tag{1}$$

$$\mathit{MGT} = \sum (F.X) \div \sum F \tag{2}$$

In this equation, *MGT*: mean germination time (day), F: the number of new germinated seeds per day of the counting day X and X days of counting. For calculating the seed vigor index, equations (3) and (4) were used (Vashisth & Nagarajan, 2010).

(3) *Vigor index I* = germination percentage × seedling length in cm (shoot + seminal root)

(4) *Vigor index II* = germination percentage × seedling dry mass in mg (shoot + seminal root)

Data from the experiment was first processed in Excel spreadsheet software, then statistical analysis of the data was done by MSTAT-C software. Means were compared by Duncan's Multiple range test.

3 RESULTS AND DISCUSSION

The results of analysis of variance showed that the application of different concentrations of titanium dioxide nanoparticles had a significant effect on all traits tested (Table 1). Also, different cadmium sulfate contents had a significant effect on other traits. The interaction between titanium dioxide nanoparticles and cadmium sulfate was significant on the rest of the studied traits except for the mean germination time.

The results showed that all concentrations of titanium dioxide increased the seed germination percentage of lentil compared to control which increased 12.4 % relative to the control (Table 2). The application of 300 ppm of titanium dioxide nanoparticles also improved the mean germination time of lentil seeds by 39 % (Table 2). Seed germination rate was from 14.75 seeds per day in the control to 23.9 seeds per day was applied in the treatment of 300 ppm of titanium dioxide nanoparticles which was an increase of 62 % compared to the control. It seems that the nanoparticles have had a positive effect on the rate of water and oxygen exchange from the seed shell to different parts of the seed and ultimately significantly improved seed germination rate.

According to the study conducted by Feizi et al. (2013) on fennel (*Foeniculum vulgare* L.), it was observed that, treatment of 60 mg l^{-1} titanium dioxide nanoparticles greatly improved the germination percentage and germination rate. But the treatment of 40 ppm of non-nanoparticles increased the mean germination time by 31.8 %. Also, other experimental results on the sage (*Salvia officinalis* L.) have shown that the concentration of 60 ppm of nanoparticles and non-nanoparticles of titanium dioxide improved germination percentage, and mean germination time compared to control. Furthermore, they declared that the germination rate in the treatment with 60 ppm nanoparticles of titanium dioxide was increased (Feizi et al., 2013). In the investigation of the effect of various concentrations of titanium dioxide nanoparticles on germination traits of silybum (*Silybum marianum* L.), Agheli et al. (2016) stated that the highest seed germination percentage was observed in the interaction of titanium dioxide nanoparticles treatment at a concentration of 100 ppm and magnetic field intensity of 30 mTesla (mT) and the application of titanium dioxide nanoparticles with a concentration of 50 ppm and a magnetic field intensity of 60 mT. These treatments improved the germination percentage by 7.5 and 6.5 times the control treatment.

The effect of different concentrations of titanium dioxide nanoparticles on the growth traits of seedling was different so that the shoot length was increased at lower concentrations, but the application of higher concentrations like 300 ppm resulted in increasing the shoot length. Nanoparticles at the concentration of 100 ppm resulted in a significant increase in shoot length from 2.273 cm to 2.623 cm. Nevertheless, application of 300 ppm concentration did not have affect its increase (Table 2). The different response of seminal root length was that only 300 ppm concentration caused a significant increase in seminal root length than in the control and increased its value by 26.2 %.The concentrations of titanium dioxide nanoparticles (100 and 300 ppm) significantly increased the seedling length compared to the control. The highest seedling length in the 100 ppm nanoparticle treatment was 12.9 % and increased its length from 4.04 cm to 4.56 cm (Table 2, Figure 2).

Agheli et al. (2016) stated that the maximum seedling length of *Silybum marianum* was observed in the application of titanium dioxide nanoparticle treatment with a concentration of 100 ppm and a magnetic field intensity of 30 mT, and also the application of titanium dioxide nanoparticles with a concentration of 50 ppm and a magnetic field intensity of 60 mT. However, application of titanium dioxide nanoparticles had the highest significant effect on seminal root mass of lentil. So that application of concentration of 100 and 300 ppm caused a significant increase of 34 % and 43 % respectively. The lowest seminal root mass was obtained in the control and 200 ppm treatment. Titanium dioxide nanoparticles had no significant effect on shoot and seedling mass (Table 2). In the investigation of the effect of titanium dioxide particles on germination of wheat it was observed that, all treatments had significant effect on seminal root mass and the highest root mass was observed in treatments of two and 50 ppm of non-nanoparticles and 100 ppm of titanium dioxide nanoparticles (Feizi, 2012).

The researchers showed that the effect of titanium dioxide nanoparticles on dry biomass was significant at 5 % probability level and reported the highest average (21481.4 kg ha^{-1}) for biological yield for titanium dioxide nanoparticles and the lowest for control (17782.4 kg ha^{-1}) (Moaveni et al., 2011a).

Seed vigor 1 was significantly affected by titanium dioxide nanoparticles and all concentrations significantly increased it compared to the control and increased its value by about 20 %. But seed vigor 2 was significant-

Table 1: Analysis variance of effects of TiO_2 nanopartcles and $CdSO_4$ on lentil seed and seedling traits

Source of Variation	df	Shoot length	Root Length	Seedling Length	Shoot mass	Root mass	Seedling mass	MGT	Germination percent	Germination rate	Vigor index 1	Vigor index 2
Nano TiO_2 (A)	3	0.392*	0.807*	1.314*	104.983*	3.473**	76.068	2.190*	384.766**	269.129**	20393.794**	1117827.3*
Cd (B)	3	13.195**	106.481**	162.166**	573.845**	92.118**	783.060**	1.219*	1049.349**	243.520**	1635187.897**	15230258.6**
AB	9	0.265*	7.412**	5.9780**	154.988*	4.228**	144.874*	0.158^{ns}	196.571**	27.322*	62836.241**	1411315.1*
Error	48	0.126	0.221	0.400	90.104	0.356	91.951	0.720	37.891	15.822	2932.194	684457.8
CV		14.67%	24.11%	14.46%	21.76%	23.18%	20.75%	65.05%	6.63%	19.15%	12.89%	19.11%

*,** denotes significant in 1 and 5 % probability level; ns denotes not significant

Table 2: Effects of TiO_2 nanopartcles concentrations on lentil seed and seedling traits

TiO_2 concentration (ppm)	Shoot length	Root length (cm)	Seedling length (cm)	Shoot mass (mg)	Root mass (mg)	Seedling mass (mg)	MGT (day)	Germination rate (seed / day)	Germination (%)	Vigor index 1	Vigor index 2
0	2.273 ^{b}	1.764 ^{b}	4.036 ^{b}	44.94 ^{a}	2.144 ^{b}	47.09 ^{a}	1.388 ^{ab}	14.75 ^{b}	85.63 ^{b}	372.9 ^{b}	4131 ^{a}
100	2.623 ^{a}	2.035 ^{ab}	4.658 ^{a}	40.66 ^{a}	2.875 ^{a}	43.54 ^{a}	1.065 ^{b}	21.88 ^{a}	94.38 ^{a}	447.9 ^{a}	4141 ^{a}
200	2.469 ^{ab}	1.768 ^{b}	4.238 ^{ab}	46.43 ^{a}	2.213 ^{b}	48.64 ^{a}	1.803 ^{a}	22.55 ^{a}	96.25 ^{a}	412.2 ^{a}	4696 ^{a}
300	2.330 ^{b}	2.226 ^{a}	4.556 ^{a}	42.49 ^{a}	3.069 ^{a}	45.56 ^{a}	0.9924 ^{b}	23.90 ^{a}	95.31 ^{a}	447.8 ^{a}	4351 ^{a}

*Means with the same letter are not significantly different at the 5 % probability.

Figure 2: Root and shoot lengths of lentil seedling in TiO_2 nanoparticles concentrations
The left Petri dish shows control,
Petri dish in center is 100 ppm T_iO_2 nanoparticles,
The right petri dish is 200 ppm T_iO_2 nanoparticles in the medium.

ly affected by the concentrations of titanium dioxide nanoparticles (Table 2).

The results of exposing seeds to cadmium sulfate showed that, with increasing concentration of cadmium sulfate in growth medium, most traits showed a decreasing trend. The germination percentage of lentil seeds in control and 100 ppm concentration treatments was the highest (97.2 and 99.4 % respectively) and decreased significantly with increasing cadmium sulfate concentrations and, the lowest is obtained at the concentration of 500 ppm (81.3 %). Also, mean germination time in the control group showed the lowest amount and at the concentration of 500 ppm of cadmium sulfate reaches the highest value (1.667) (Table 3).

According to Shafiq et al. (2008), decrease in germination of seeds could be due to the decomposition of food stored in the seeds as a result of application of cadmium. According to studies on the effect of heavy metals on maize germination, Parmon et al. (2014) opined that heavy metals had a significant effect on maize germination and seed strength. Low levels of metals increase the germination percentage (from 93 % to 98 % in lead nitrate, from 93 % to 97 % in bismuth nitrate and from 94 % to 96 % in cadmium) and seed strength (from 1200 to 1606 in bismuth nitrate and from 1568 to 1592 in lead nitrate), but high levels reduced both parameters. Mahmood et al. (2005) also stated that different levels of copper had no significant effect on maize germination but reduced the early growth of maize.

Application of cadmium sulfate at a concentration of 500 ppm compared to the control reduced the germination rate by 37 %. However, the effect of concentrations of 100 and 250 ppm on germination rate was not significant (Table 3). According to the results, it seems that lentil seeds can tolerate up to 100 ppm of cadmium sulfate in these conditions but higher concentrations cause toxicity for this plant.

The results obtained by Vasilev (2003) about the trait of *Hordeum vulgare* L. on different levels of cadmium showed that, the maximum effect on reducing growth is related to treatment of 10 mg l^{-1} of cadmium. The maximum reduction in growth was about 20 percent compared to control treatment.

The application of 100 ppm of cadmium sulfate increased the shoot length compared to the control from 2.876 cm to 3.264, and then at concentrations of 250 and 500 ppm significantly reduce it by 17.1 and 59.2 percent (Table 3, Figure 3). But seminal root length showed a significant decrease with increasing concentration of heavy metals. Seminal root length was decreased by 69, 92 and 100 %, respectively at the concentrations of 100, 250 and 500. In other words, the effect of cadmium sulfate toxicity on seminal root was much more than that of the shoot length, since the seminal root was directly exposed to stress, and it had the most contact with this.

The concentration–response phenomenon that is characterized by low-dose stimulation and high-dose inhibition is called as hormesis or hormetic effect. An increased number of studies has described the improved performance of some plant species to Cd exposure, despite Cd accumulation in their roots and shoots. These results designate that plants have developed protective strategies to neutralize the side-effects from Cd toxicity or, more controversially, mechanisms that employ Cd as beneficial element (Carvalhoa et al. 2020).

Carvalhoa et al. (2020) reported that carbonic anhy-

Table 3: Effects of $CdSO_4$ concentrations on lentil seed and seedling traits

Cd concentration (ppm)	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Shoot mass (mg)	Root mass (mg)	Seedling mass (mg)	MGT (day)	Germination rate (seed / day)	Germination (%)	Vigor index 1	Vigor index 2
0	2.876 ^{b}	5.659 ^{a}	8.535 ^{a}	41.29 ^{bc}	5.262 ^{a}	46.56 ^{a}	0.9996 ^{b}	24.24 ^{a}	97.19 ^{ab}	831.4^{a}	4531 ^{b}
100	3.264 ^{a}	1.716 ^{b}	4.980 ^{b}	48.03 ^{ab}	3.825 ^{b}	51.86 ^{a}	1.245 ^{ab}	22.27 ^{a}	99.38 ^{a}	494.4 ^{b}	5155 ^{a}
250	2.382 ^{c}	0.4175 ^{c}	2.799 ^{c}	48.95 ^{a}	1.212 ^{c}	50.16 ^{a}	1.337 ^{ab}	21.36 ^{a}	93.75 ^{b}	261.9^{c}	4712 ^{ab}
500	1.174 ^{d}	0.000 ^{d}	1.173 ^{d}	36.25 ^{c}	0.000 ^{d}	36.25 ^{a}	1.667 ^{a}	15.20 ^{b}	81.25 ^{c}	93.14^{d}	2920 ^{c}

*Means, in each column, followed by same letter are not significantly different at the 5 % probability level- using Duncan´s Multiple Range Test.

Table 4: Interaction effects of TiO_2 nanoparticles and $CdSO_4$ concentrations on lentil seed and seedling traits

Nano TiO_2 (ppm)	$CdSO_4$ (ppm)	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Shoot Weight (mg)	Root mass (mg)	Seedling mass (mg)	MGT (day)	Germination rate (seed/day)	Germination (%)	Vigor index 1	Vigor index 2
0	0	3.113 ^{abc}	3.128 ^{d}	6.240 ^{c}	41.60 ^{bc}	3.600 ^{cd}	45.20 ^{abc}	1.095 ^{a}	20.00 $^{c-g}$	93.75 ^{abc}	582.7 ^{d}	4271 ^{bcd}
	100	2.895^{abc}	3.928 ^{c}	6.823 ^{c}	49.05 ^{ab}	4.975 ^{b}	54.03 ^{ab}	1.405 ^{a}	16.13 ^{fg}	98.75 ^{a}	673.4 ^{c}	5343 ^{ab}
	250	1.928 ^{e}	0.000 ^{f}	1.928 ^{ef}	49.30 ^{ab}	0.000 ^{f}	49.30 ^{abc}	1.342 ^{a}	14.53 ^{g}	91.25 ^{abc}	176.1 ^{hi}	4505 ^{abc}
	500	1.155 ^{f}	0.000 ^{f}	1.155 ^{f}	39.83 ^{bc}	0.000 ^{f}	39.83 ^{bcd}	1.709 ^{a}	8.338 ^{h}	58.75 ^{d}	59.58 ^{j}	2405 ^{e}
100	0	3.173 ^{ab}	6.105 ^{b}	9.278 ^{ab}	42.10 ^{bc}	5.550 ^{b}	47.65 ^{abc}	0.8823 ^{a}	26.92 ^{a}	97.50 ^{ab}	903.3 ^{b}	4641 ^{abc}
	100	3.347 ^{a}	0.9250 ^{e}	4.273 ^{d}	47.70 ^{ab}	3.250 ^{cd}	50.95 ^{ab}	0.9313 ^{a}	20.29 b-g	100.0 ^{a}	427.3 ^{ef}	5095 ^{abc}
	250	2.722 ^{bcd}	1.110 ^{e}	3.832 ^{d}	46.13 ^{ab}	2.700 ^{de}	48.83 ^{abc}	0.8823 ^{a}	25.42 $^{a-d}$	93.75 ^{abc}	354.7 ^{f}	4577^{abc}
	500	3.367 ^{a}	0.000 ^{f}	1.250 ^{f}	26.73 ^{c}	0.000 ^{f}	26.73 ^{d}	1.562 ^{a}	14.88 ^{g}	86.25 ^{c}	106.3 ^{ij}	2249 ^{e}
200	0	2.595 ^{cd}	5.960 ^{b}	8.613 ^{b}	40.75 ^{bc}	5.500 ^{b}	46.25 ^{abc}	1.158 ^{a}	21.96 $^{a-g}$	97.50 ^{ab}	838.4 ^{b}	4502 ^{abc}
	100	3.367 ^{a}	1.112 ^{e}	4.480 ^{d}	50.08 ^{ab}	3.350 ^{cd}	53.42 ^{ab}	1.868 ^{a}	25.92 ^{abc}	98.75 ^{a}	445.6 ^{e}	5279 ^{abc}
	250	2.565 ^{cd}	0.000 ^{f}	2.595 ^{e}	60.85 ^{a}	0.000 ^{f}	60.85 ^{a}	2.085 ^{a}	23.91 $^{a-e}$	96.25 ^{abc}	250.4 ^{gh}	5871 ^{a}
	500	1.263 ^{f}	0.000 ^{f}	1.263 ^{f}	34.05 ^{bc}	0.000 ^{f}	34.05 ^{cd}	2.104 ^{a}	18.41 ^{efg}	92.50 ^{abc}	117.4 ^{ij}	3131 ^{de}
300	0	2.565 ^{f}	7.445 ^{a}	10.01 ^{a}	40.72 ^{bc}	6.400 ^{a}	47.13 ^{abc}	0.8625 ^{a}	28.10 ^{a}	100.0 ^{a}	1001 ^{a}	4713 ^{abc}
	100	3.445 ^{a}	0.90 ^{e}	4.345 ^{d}	45.30 ^{b}	3.725 ^{c}	49.03 ^{abc}	0.7750 ^{a}	26.75 ^{ab}	100.0 ^{a}	434.5 ^{ef}	4903^{abc}
	250	2.283 ^{de}	0.560 ^{ef}	2.842 ^{e}	39.53 ^{bc}	2.150 ^{e}	41.67 ^{bc}	1.040 ^{a}	21.58 $^{a-f}$	93.75 ^{abc}	266.3 ^{g}	3895 ^{cd}
	500	1.028 ^{f}	0.000 ^{f}	1.025 ^{f}	44.40 ^{b}	0.000 ^{f}	44.40 ^{bc}	1.292 ^{a}	19.19 $^{d-g}$	87.50 ^{bc}	89.22 ^{j}	3895 ^{cd}

*Means, in each column, followed by same letter are not significantly different at the 5 % probability level- using Duncan´s Multiple Range Test.

drase activity was also increased in bean plants (*Phaseolus vulgaris* L.) under exposure to low Cd concentration, despite slight decreases in the amount of this enzyme protein. The results of Ahmadvand et al. (2012) showed that, cadmium treatment reduced the mean of seminal root and shoot length and root to stem (R/S) ratio by 96.83 %, 29.66 %, and 27.52 %, respectively compared to control. Similar to seminal root length, the length of the lentil seedlings significantly decreased with increasing cadmium sulfate concentration. The maximum length of seedling was 8.54 cm in control and less in the treatment of 500 ppm at 1.17 cm (Equivalent to 86 % of reduction).

Based on experiments conducted on rice plant, the length of aerial organs, plant biomass and water content decreased due to copper pollution. So that at concentrations higher than 0.5, the formation of the root and at a concentration of more than 1.5 mM, the formation of the leaf and shoot was stopped (Ahsan et al., 2007).

Also, in a study on *Helianthus annuus* (L.), the cause of the decrease in seedlings length in the presence of elements such as nickel, copper, cadmium and zinc attributed to the decrease in cellular development. In the existence of heavy metals, plant meristem cells, and lean and endosperm enzymes seems to be reduced, and the cells begin to digest food stores transferred to the primary root and above ground organs in the form of a solution. Therefore, the activity of hydrolytic enzymes is affected when the substrates cannot reach the root and the aerial organs; therefore, it affects the length of the seedling (Houshmandfar & Moraghebi, 2011).

Also, the highest shoot mass was observed in 250 ppm Cd concentration treatment with 48.95 mg and the lowest in 500 ppm treatment with 36.25 mg, which decreased by 12 % compared to control. However, the seminal root mass was more negatively affected by the increased concentration of cadmium sulfate and showed a decreasing trend. The highest seminal root mass was observed in control and lowest in 500 ppm Cd concentration. The total seedling mass was associated with the treatment of 500 ppm of cadmium sulfate which was lower than control by 22 % (Table 3). Similar trends were observed in previous studies with respect to cadmium contamination. Moteshare Zadeh et al. (2014) concluded that the highest dry mass of bean shoots (2.25 g in a pot) was obtained in treatment of 0 ppm of cadmium and the lowest mass (1.59 g in a pot) was obtained at 80 ppm of cadmium. With increasing cadmium concentration, shoot dry mass decreased. With increasing cadmium concentration, root dry mass in the treatment of 80 mg of cadmium was lower than treatment of 40 mg. In studying the accumulation of cadmium and lead in shoots of *Helianthus annuus*, castor, alfalfa and mustard plants in the hydroponic culture, the researchers showed that the biomass of plants decreased with increasing concentration of cadmium and lead (Zhi Xin, et al, 2007).

Increasing the concentration of cadmium sulfate caused a significant decrease in both parts of the seed vigor index and showed the lowest value in both traits (Table 3). According to Parmon et al. (2014), seed strength was also affected by heavy metals. Comparison of mean values indicated that with increasing nitric acid concentration, seed strength was constant and it significantly decreased by reaching the amount of 1000 ppm. Increasing the concentration of cadmium caused a sharp decrease in seed vigor.

The production of active radicals and lipid peroxi-

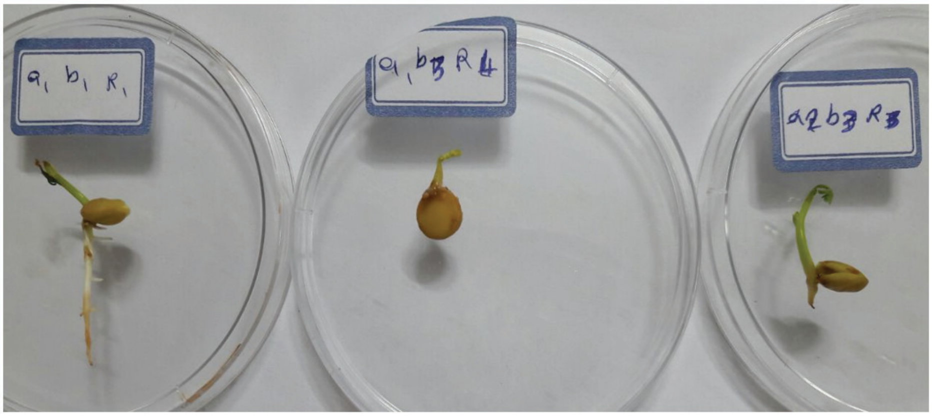


Figure 3: Root and shoot lengths of lentil seedling in TiO_2 nanoparticles and cadmium concentrations.
The left Petri dish shows control,
Petri dish in center is 0 ppm T_iO_2 nanoparticles +250 ppm cadmium
The right Petri dish is 100 ppm T_iO_2 nanoparticles+250 ppm cadmium in the medium.

dation, due to oxidative stress, disrupt the function of the cell membrane and eliminate the permeability of its selection, this will increase the leakage of seeded materials and reduce germination and seed vigor. Oxidative stress also changes the activity of antioxidant enzymes (Hussain et al., 2011).

Application of titanium dioxide nanoparticles could significantly reduce the negative effects of cadmium stress and improve lentil germination percentage. In the absence of any nanoparticles, germination percentage of seeds in the treatment of 500 ppm cadmium sulfate was 58.75 %. However, using 100 ppm of titanium dioxide nanoparticles (at the same concentration of 500 ppm of cadmium sulfate) germination percentage was 86.25 % which showed an increase of 46.8 % relative to the non-application of nanoparticles (Table 4). Such an upward trend was observed in the application of 200 ppm and 300 ppm cadmium concentrations. Under contamination of 200 ppm and 300 ppm cadmium sulphate, titanium dioxide nanoparticles application with a concentration of 200 % increased germination percent by 92.5 %. The mutual effect of the application of titanium dioxide nanoparticles and cadmium sulfate on mean germination time was not significant.

The use of titanium dioxide nanoparticles significantly prevent the reduction of the germination rate due to the stress of cadmium sulfate in lentil seeds. In the non-application of nanoparticles, cadmium stress at a concentration of 500 ppm of cadmium sulfate caused a 58 % decrease in germination rate. While in this concentration, the use if 100 ppm nanoparticles of titanium dioxide reduced the germination rate by only 44, 16 and 31.7 %, respectively (Table 4). Therefore, impregnating our seed with 200 ppm nanoparticles of titanium dioxide before planting can improve the germination rate by 120 % compared to the non-application of nanoparticles under stress conditions of 500 ppm of cadmium sulfate. Therefore, the reduction of the negative effects of heavy metal stress such as cadmium by titanium dioxide nanoparticles, can be considered as a very useful and valuable solution and, this potential is benefited in order to control and reduce such stresses.

The interaction of titanium dioxide nanoparticles and cadmium sulfate on hypocotyl, seminal root and seedling length was significant. The highest shoot length was observed in the treatment of 100 ppm nanoparticles and 500 ppm cadmium sulfate, 200 ppm nanoparticles combined with 100 ppm cadmium sulfate and 300 ppm nanoparticles plus 100 ppm sulfate cadmium. In the conditions of the highest cadmium sulfate stress (500 ppm), the use of titanium dioxide nanoparticles generally prevented the effect of heavy metal stress and the shoot length remained unchanged compared to the control (3.17 cm in the control and 3.37 in the 500 ppm of cadmium sulfate and 100 ppm nanoparticles of titanium dioxide). Soltani et al. (2014) provided the results of comparison between the interactions showing that the concentration of titanium dioxide nanoparticles was 0.02 % of foliar application and, the highest grain yield was at the 60 % of shoot and the concentration of titanium dioxide nanoparticles was 0.02 % of foliar application. Also, in flowering stage, it had the lowest grain yield.

4 CONCLUSION

The current study results clearly demonstrated the capacity of TiO_2 nanoparticles treatments to ameliorate the tolerance to Cd stress compared to a non-stress condition. The capability of TiO_2 nanoparticles treatments to maintain and increase the seed germination percent and seedling characteristics of lentil. The decreased toxicity of Cd was more likely attributed to the decreased bioavailability of free Cd ion owing to adsorption and speciation change of Cd in the presence of TiO_2 nanoparticles. The present study demonstrates that TiO_2 nanoparticles treatments can be applied to mitigate the negative impact of Cd stress on different pulse crops like lentil in contaminated areas with heavy metals, particularly cadmium. Nevertheless, the interaction between TiO_2 nanoparticles and co-existing pollutants in the environment remains uncertain; risk assessment studies have been unconvincing due to the tremendously complicated environmental conditions. Although this study demonstrated the potential of nanoparticles for ameliorating of heavy metal, further exploration and investigation is needed to elucidate and develop these possibilities.

5 ACKNOWLEDGEMENT

This work has been supported by the research deputy of Education and Research, University of Torbat Heydarieh, Iran, that the authors hereby acknowledge. The grant number is 11.

6 REFERENCES

Agheli, N., Aliakbarkhani, Z., Behnam, A., Feizi, H. (2016). Stimulation of silybum seed germination and seedling growth by TiO_2 nanoparticles and magnetic field. *First National Conference on Medicinal Plants,* University of Gonbad Kavus, Iran. (In Persian).

Ahmadvand, S., Bahmani, R. Habibi, D., Forozesh, P. (2012). Study on the effect of cadmium chloride on growth parameters and some physiological traits in bean seedlings. *Plant*

Breeding and Agronomy Journal. 8, 165-182 (In Persian with English Abstract).

Ahsan, N., Lee, D.G., Lee, S.H., Kang, K.Y., Lee, J.J., Kim, P.J., Yoon, H.S., and Lee, H.B. (2007). Excess copper induced physiological and proteomic changes in germinating rice seeds. *Chemosphere*. *67*(6), 1182–1193. https://doi.org/10.1016/j.chemosphere.2006.10.075

Carvalhoa M. E.A., Castro, P. RC., Azevedo, R. A. (2020). Hormesis in plants under Cd exposure: From toxic to beneficial element? *Journal of Hazardous Materials. Volume 384* https://doi.org/10.1016/j.jhazmat.2019.121434

Chen, J., Qian, Y., Li, H., Cheng, Y., Zhao, M., (2015). The reduced bioavailability of copper by nano-TiO2 attenuates the toxicity to *Microcystis aeruginosa*. *Environmental Science Pollution Research, 22*, 12407–12414. https://doi.org/10.1007/s11356-015-4492-9

Deng, R., Lin, D., Zhu, L., Majumdar, S., White, J.C., Gardea-Torresdey, J.L., Xing, B. (2017). Nanoparticle interactions with co-existing contaminants: joint toxicity, bioaccumulation and risk. *Nanotoxicology. 11*(5), 591-612. https://doi.org/10.1080/17435390.2017.1343404

Dolatabadi, A., Sani, B., and Moaveni P. (2015). Impact of nanosized titanium dioxide on agronomical and physiological characteristics of annual medic (*Medicago scutellata* L.). *Cercetări Agronomice în Moldova, 48*, 53-61. https://doi.org/10.1515/cerce-2015-0041

Engates, K.E., Shipley, H.J. (2011). Adsorption of Pb, Cd, Cu, Zn, and Ni to titanium dioxide nanoparticles: effect of particle size, solid concentration, and exhaustion. *Environmental Science Pollution Research, 18*, 386–395. https://doi.org/10.1007/s11356-010-0382-3

Feizi, H. and Rezvani Moghaddam, P. (2011). Influence of magnetic field and silver nanoparticles in comparison to macro and micro nutrient fertilizers on growth, yield and silage quality of maize. *Journal of Water and Soil, 24,* 1062-1072. (In Persian with English Abstract).

Feizi, H. (2012). *The role of metallic nanoparticles (TiO_2, Fe_2O_3) and magnetic field on germination, nutrient absorption, growth and quantitative and qualitative characteristics of wheat*. PhD. Thesis. Ferdowsi University of Mashhad, Iran. (In Persian with English Abstract).

Feizi, H., Kamali, M., Jafari, L. & Rezvani Moghaddam, P. (2013). Phytotoxicity and stimulatory impacts of nanosized and bulk titanium dioxide on fennel (*Foeniculum vulgare* Mill). *Chemosphere, 91,* 506–511. https://doi.org/10.1016/j.chemosphere.2012.12.012

Houshmandfar, A., and Moraghebi, F. (2011). Effect of mixed cadmium, copper, nickel and zinc on seed germination and seedling growth of safflower. *African Journal of Agricultural Research, 6*(5), 1182-1187.

Hussain, S.S., Ali, M., Ahmad, M., and Siddique, K.H.M. (2011). Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. *Biotechnology Advances, 29*(3), 300-311. https://doi.org/10.1016/j.biotechadv.2011.01.003

ISTA. (2009). *ISTA Rules*. International Seed Testing Association. Zurich, Switzerland. 47 pp.

Mahmood, S., Hussain, A., Zaeed, Z., and Athar, M. (2005). Germination and seedling growth of corn *(Zea mays* L.) under varying levels of copper and zinc. *International Journal of Environmental Science Technology, 2*(3), 269-274. https://doi.org/10.1007/BF03325886

Maguire, I. D. (1982). Speed of germination – Aid in selection and evaluation for seedling emergence and vigor. *Crop Science, 22*, 177–176. https://doi.org/10.2135/cropsci1962.0011183X000200020033x

Miao, W., Zhu, B., Xiao, X., Li, Y., Dirbaba, N.B., Zhou, B., Wu, H. (2015). Effects of titanium dioxide nanoparticles on lead bioconcentration and toxicity on thyroid endocrine system and neuronal development in zebrafish larvae. *Aquatic Toxicology, 161*, 117-26. https://doi.org/10.1016/j.aquatox.2015.02.002

Moaveni, P., Talebi, R., Farahani, H.A. and Maroufi, K. (2011). Effect of TiO_2 nanoparticles spraying on barley (*Hordem vulgare* L.) under field condition. *Advances Environmental Biology, 5*(7), 1663-1667.

Motesharezade, B., Aghaee, L., Savaghebi, G. (2014). Influence of Cd and Pb application on their absorption and growth of two bean cultivars. *Environmental Stress in Crop Science, 7,* 257-271. (In Persian with English Abstract).

Pais, I. (1983). The biological importance of titanium. *Journal of Plant Nutrition, 6*, 3-131. https://doi.org/10.1080/01904168309363075

Parmoon, G., Ebadi, A., Ghaviazm A., and Miri, M. (2013). Effect of seed priming on germination and seedling growth of Chamomile under salinity. *EJCP, 6*(3), 145-164. (In Persian with English Abstract).

Parmoon, G. Ebadi, E. Ghahremani, M. Moosavi, S.A. (2014). Influence of heavy metal on seed germination and vigor of maize in control condition. *Journal of Seed Research, 4*, 40-51. (In Persian with English Abstract).

Shafiq M., Iqbal, M.Z. and Athar M. (2008). Effect of lead and cadmium on germination and seedling growth of *Leucaena leucocephala. Journal of Science Environmental Management, 12*(2), 61- 66.

Soltani, M., Moaveni, P. Noori H. (2014). Investigation of foliar application effect of TiO2 nanoparticles on yield and yield component of lentil. *Iranian Journal Plant Ecophysiology Research, 9,* 78-88. (In Persian with English Abstract).

Tan, C., Wang, W.X., (2014). Modification of metal bioaccumulation and toxicity in *Daphnia magna* by titanium dioxide nanoparticles. *Environmental Pollution, 186*, 36–42. https://doi.org/10.1016/j.envpol.2013.11.015

Vashisth, A., and Nagarajan, S. (2010). Effect on germination and early growth characteristics in sunflower (*Helianthus annuus*) seed exposed to static magnetic field. *Journal of Plant Physiology, 167,* 149-156.

Vassilev, A. (2003). Barley seedlings as bio-indicators for water contamination by cadmium. *Journal of Environmental Protection and Ecology, 4*(2), 354-360. https://doi.org/10.1016/j.jplph.2009.08.011

Zhi-Xin, N., Li-na, S., Tie-heng, S., Yu-shuang L., and Hong, W. (2007). Evaluation of phytoextracting cadmium and lead by sunflower, ricinus, alfalfa and mustard in hydroponic culture. *Environmental Sciences, 19*, 961-967. https://doi.org/10.1016/S1001-0742(07)60158-2

doi:10.14720/aas.2020.116.1.1722

Short original scientific article / kratek izvirni znanstveni članek

Genetic variability in morpho-agronomic traits of various accessions of *Vigna subterranea* (L.) Verdc.

Maruf Olaide YEKEEN[1,2], Hafsoh Olajumoke SHAIB-RAHIM[3], Joy OLABODE[3]

Received June 09, 2020; accepted June 17, 2020.
Delo je prispelo 09. junija 2020, sprejeto 17. junija 2020.

Genetic variability in morpho-agronomic traits of various accessions of *Vigna subterranea* (L.) Verdc.

Abstract: *Vigna subterranea* is an indigenous African legume widely cultivated across the continent. It is a highly diverse crop exhibiting diversity in morpho-agronomic characteristics. This genetic diversity is also crucial for crop improvement as it determines which breeding strategy/ methodology to utilize in its improvement. The study evaluated the diversity in morpho-agronomic characteristics of different accessions of Bambara groundnut. 'TVSu596' exhibited the highest germination percentage (100 %) and shortest days to 50 % flowering (40 days) while 'TVSu11' had the highest number of leaves (146). Qualitative evaluation revealed the diversity existing in their morphological characteristics. Elemental analysis showed the accessions are rich in mineral elements and exhibit diversity. Principal component analysis revealed 83.37 % of total variations in morphological and yield traits by the first three principal axes. Traits such as number of leaves, petiole length, and days to 50 % flowering furnish most of the variations. Cluster analysis revealed the accessions clustered into 4 groups. These accessions are therefore recommended for utilization in development of improved cultivars or early-maturing varieties

Key words: *Vigna subterranea*; Bambara groundnut; genetic variation; morpho-agronomic analysis; chemical analysis

Genetska raznolikost in morfološko-agronomske lastnosti različnih akcesij bambare (*Vigna subterranea* (L.) Verdc.)

Izvleček: Bambara (*Vigna subterranea*) je samonikla afriška stročnica, ki se na veliko goji širom kontinenta. Je zelo raznolika poljščina z zelo različnimi morfološko-agronomskimi lastnostmi. Ta genetska raznolikost je najpomembnejša za njeno izboljšanje in določa tudi žlahtniteljske strategije in metode, ki jih je potrebno pri tem uporabiti. V raziskavi je bila ovrednotena raznolikost morfološko agronomskih lastnosti različnih akcesij bambare. Akcesija TVSu596 je pokazala največji odstotek kalivosti (100 %) in najkrajše obdobje do 50 % cvetenja (40 dni), akcesija TVSu11 je imela največje število listov (146). Kakovostno ovrednotenje je odkrilo, da obstajajo razlike v morfološko-agronomskih lastnostih. Snovna analiza je pokazala, da so akcesije bogate na mineralih in da so razlike v vsebnostih le teh velike. Analiza glavnih komponent je odkrila, da je 83,37 % celotne variabilnosti v morfoloških lastnostih in je pojasnjeno s prvimi tremi osnovnimi osmi. Lastnosti kot so število listov, dolžina peclja in število dni do 50 % cvetenja prispevajo največji delež variabilnosti. Analiza združevanja je odkrila, da so akcesije porazdeljene v 4 skupine, ki jih priporočamo za uporabo pri vzgoji izboljšanih sort ali za zgodnje dozorevanje.

Ključne besede: *Vigna subterranea*; bambara; genetska raznolikost; analiza morfološko-agronomskih lastnoti; kemijska analiza

---

[1] The Federal Polytechnic, Ile-Oluji, Nigeria
[2] Corresponding author, e-mail: Yekeenolaidem@gmail.com
[3] Federal College of Forestry, Ibadan, Nigeria

1 INTRODUCTION

Vigna subterranea (L.) Verdc. popularly known as bambara groundnut is an indigenous African legume crop which is widely cultivated across the continent (Mkandawire, 2007). The seeds are used during the performance of funeral rites. It also possess medicinal properties, being chiefly used in the control of diarrhoea, skin rashes, and swollen jaw disease (Akpalu et al., 2013). *V. subterranea* is a highly diverse crop in terms of its size of seeds, colour of seeds, plant height, eye colour, eye patterns, hilum colour and patterns, and other morpho-agronomic characteristics (Ouedraogo et al., 2008; Akpalu et al., 2013). This genetic diversity is the basis for adaptation and survival of the species and makes it easy for them to adapt to changing of environmental conditions (Rao et al., 2002; Sevik et al., 2010). Adequate information about genetic diversity within crop species is essential for crop improvement as it determines methodologies to utilize in the improvement of the species (Aliyu et al., 2016). Hence, estimation of genetic variability of different traits is not only of economic importance but also crucial in cultivar development and improvement of any plant species (Odongo et al., 2015). This study therefore comparatively assessed the genetic variability in the morpho-agronomic traits and mineral elements present in seeds of seven colour variants of *V. subterranea*.

2 MATERIALS AND METHODS

The study was carried out within Federal College of Forestry, Ibadan, Nigeria. Seeds of seven accessions of *V. subterranea* (TVSu 119, TVSu 596, TVSu 182, TVSu 11, TVSu 367, TVSu 49 and TVSu 254) were utilized in the study. Germination test was conducted in a laboratory and the seedlings were later transplanted into modified plastic buckets arranged in a randomized complete block design (RCBD) with three replications. For the mineral elements determination, seeds of each accession were ground and 1 g of each was dry ashed for 5 h in a muffle furnace at 550 °C until a white residue with constant mass was obtained. The ash was digested with 3M HCl and some of the mineral elements were investigated using atomic absorption spectrophotometer (AAS) (AOAC, 2003). For the evaluation of quantitative data, the analysis of variance (ANOVA), PCA and correlations were used.

3 RESULTS AND DISCUSSION

The result of elemental analysis is presented in Table 1. The result showed that 'TVSu119' is a good source of mineral elements though it is a little deficient in copper content, followed by 'TVSu596' which is rich in magnesium and potassium. This result is similar to that observed by Amarteifio et al. (2006).

Qualitative characteristics assessment result showed that two types of petiole pigmentation pattern (non-pigmented and slightly-pigmented) were observed. Leaf colour assessment result also showed the presence of three different types (dark green, pale green and green) of pigmentation patterns. The result of quantitative characters studied on the seven accessions is presented in Table 2. Result showed that significant difference ($p \leq 0.01$) exists in all characters among all the accessions studied. NL ranged from 67.80 to 146.00. This contrasts with the result of Shegro et al. (2013). TLL ranged from 5.65 cm to 8.16 cm in 'TVSu49'. This is similar to the result of Shegro et al. (2013) but contrasted with the result of Ndiang et al. (2014).

TLW ranged from 2.38 cm to 4.72 cm in 'TVSu49'. This is similar to the result of Shegro et al. (2013). Petiole length ranges from 15.13 cm to 26.55 cm in 'TVSu49'. This contrasts with the result of Shegro et al. (2013) and Ndiang et al. (2014). DFF ranged from 40 days to 49 days. This is similar to the result of Brink et al. (2006) but contrasted with the result of Ouedraogo et al. (2008). D5F ranged from 40 days to 43 days. This is similar to the result of Brink et al. (2006). Number of seeds/plant ranged from 3.0 to 6.0. The low seed yield observed in these ac-

Table 1: Selected mineral elements studied on the analysed accessions of *V. subterranea*

Varieties	Accessions	Ca (%)	Mg (%)	K (%)	Fe (mg g^{-1})	Cu (mg g^{-1})
1	TVSu119	0.03	0.24	1.61	40.92	7.49
2	TVSu596	0.01	0.25	1.70	31.97	8.49
3	TVSu182	0.02	0.21	1.46	20.93	7.48
4	TVSu11	0.02	0.21	1.56	20.45	9.48
5	TVSu367	0.03	0.23	1.31	25.97	8.99
6	TVSu49	0.02	0.21	1.42	36.99	9.50
7	TVSu254	0.03	0.23	1.53	30.94	19.96

Table 2: Quantitative characters studied on the analysed accessions of *V. subterranea*

Accessions	GP	HL	SR	LN	NL	TLL	TLW	PL	DFF	D5F
TVSu119	56^{e}	2.25^{bc}	3.20^{b}	9.70^{b}	67.80^{d}	6.28^{c}	2.38^{c}	17.30^{bc}	49^{a}	51^{a}
TVSu596	100^{a}	4.26^{a}	8.96^{a}	17.92^{a}	92.20^{c}	5.65^{c}	2.50^{c}	15.13^{d}	40^{d}	40^{f}
TVSu182	84^{b}	2.92^{bc}	8.17^{a}	13.65^{ab}	126.90^{ab}	6.12^{c}	3.08^{b}	18.73^{bc}	45^{b}	49^{b}
TVSu11	60^{d}	2.84^{b}	4.23^{b}	13.07^{ab}	146.00^{a}	5.80^{c}	2.86^{b}	19.33^{b}	40^{d}	43^{e}
TVSu367	56^{e}	2.44^{b}	3.95^{b}	5.78^{b}	106.10^{bc}	6.77^{b}	3.01^{b}	17.05^{cd}	41^{d}	44^{e}
TVSu49	79^{c}	2.24^{bc}	4.31^{b}	8.08^{b}	85.50^{cd}	8.16^{a}	4.72^{a}	26.55^{a}	40^{d}	46^{c}
TVSu254	80^{c}	1.17^{c}	4.47^{b}	8.52^{b}	122.10^{b}	8.07^{a}	2.98^{b}	19.18^{b}	43^{c}	45^{cd}
Mean	73.57	2.50	5.33	10.96	107.66	6.69	3.08	19.04	42.57	45.43
CV (%)	1.26	30.61	38.54	49.05	21.05	9.54	8.86	11.09	2.35	2.20
S.E.M ±	0.53	0.34	0.92	2.40	7.17	0.20	8.62	0.67	0.58	0.58
F Test	*	*	*	*	*	*	*	*	*	*
L.S.D (0.05)	1.62	0.99	2.66	6.96	20.25	0.57	0.24	1.89	1.75	1.75

NOTE: GP-Germination percentage; HL-Hypocotyl length; SR-Seminal root length; LN-Lateral root number; NL-Number of leaves; TLL-Terminal leaflet length; TLW- Terminal leaflet width; PL-Petiole length; DFF-Days to first flowering; D5F-Days to 50 % flowering. Means with the same letters are not significantly different from each other. *: Significant at 5 % level of probability. **: Significant at 1 % level of probability.

Table 3: Correlations for morphological and yield characteristics of the analysed accessions of *V. subterranea*

	GP	HL	SR	LN	NL	TLL	TLW	PL	DFF	D5F
GP	1									
HL	0.45**	1								
SR	0.84**	0.73**	1							
LN	0.64**	0.81**	0.81**	1						
NL	-0.01	-0.06	0.15	0.17	1					
TLL	0.01	-0.77**	-0.45**	-0.71**	-0.15	1				
TLW	0.12	-0.28	-0.18	-0.40**	-0.07	0.70**	1			
PL	-0.01	-0.42**	-0.34*	-0.41**	-0.04	0.70**	0.94**	1		
DFF	-0.30*	-0.30*	-0.19	-0.12	-0.37*	-0.11	-0.42**	-0.25	1	
D5F	-0.39*	-0.47**	-0.34*	-0.37*	-0.30*	0.16	0.05	0.21	0.86**	

* Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level.

cessions is similar to the result of Linnemann (1995). The co-efficient of variation (CV) ranged from 1.26 % to 49.05 %. This showed that a high level of genetic diversity, exploitable by plant breeders, existed in the studied traits. Standard error of mean (SEM) ranges from 0.20 to 0.92 showing that the data is normally distributed with a steep bell-shaped curve. Pearson's correlation analysis (Table 3) showed that petiole length was strongly and positively correlated with TLW and TLL but negatively correlated with HL, LN and DFF. Positive correlation of germination percentage with SR, LN and HL indicated that accessions with higher germination percentage exhibited increased vegetative growth characteristics. While negative correlation with DFF and D5F indicated that accessions with higher germination percentage possessed lower days to first and 50 % flowering.

The results of the PCA analysis are presented in Table 4. The first five principal components explained about 98.2 % of the total variation. The Eigen values ranged from 4.33 to 0.64. Principal components axes 1 and 2 accounted for 43.33 % and 25.84 % variation respectively.

4 CONCLUSION

From the investigation, this study therefore submits that the germplasm displayed high genetic variability for the characteristics studied; hence they can still be im-

Table 4: The Eigenvalues, proportion of variability and the phenotypic traits contributing to the first five Principal components of *V. subterranea*

Item	PC1	PC2	PC3	PC4	PC5
Eigenvalues	4.33	2.58	1.42	0.84	0.64
%Variance per PC axes	0.43	0.26	0.14	0.08	0.06
Cumulative Variance	0.43	0.69	0.83	0.92	0.98
Morphological traits			Eigenvectors		
Terminal leaflet length (cm)	0.38	0.29	-	-	-
Petiole length (cm)	0.31	0.38	0.20	-	-
Days to 50 % flowering	0.26	-0.35	0.39	0.33	0.31
Terminal leaflet width (cm)	0.26	0.47	0.21	-	0.34
Days to first flowering	-	-0.52	0.36	0.25	-
Lateral root number	-0.44	-	-	-	-
Hypocotyl length (cm)	-0.42	-	-	-0.33	0.39
Seminal root length (cm)	-0.40	-	0.30	0.27	-
Germination %	-0.27	0.33	0.43	0.21	-0.40
Number of leaves	-	-	-0.55	0.74	-

NB: Only eigenvectors with values ≥ 0.20 are presented.

proved genetically, as they can be useful to plant breeders who may be interested in specific traits and their variation within the species. The earliness in days to first and 50 % flowering displayed by TVSu596 meant it could be utilized in the development of early-maturing varieties or cultivars. The study thus recommends further studies on the nutritional value of these accessions in order to evaluate their protein, carbohydrate and fat contents.

5 ACKNOWLEDGEMENT

The *Vigna unguiculata* accessions utilized in this study were obtained from Genetic Resources Centre, International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria.

6 REFERENCES

Akpalu, M. M., Atubilla, I. A., & Oppong-Sekyere, D. (2013). Accessing the level of cultivation and utilization of Bambara groundnut (*Vigna subterranea* (L.) Verdc.), in the Sumbrungu community of Bolgatanga, Upper East Region, Ghana. *International Journal of Plant, Animal and Environmental Sciences,. 3*(3), 68-75.

Aliyu, S., Massawe, F. & Mayes, S. (2016). Genetic diversity and population structure of Bambara groundnut (*Vigna subterranea* (L.) Verdc.): synopsis of the past two decades of analysis and implications for crop improvement programmes. *Genetic Resources and Crop Evolution, 63*, 925–943. https://doi.org/10.1007/s10722-016-0406-z

Amarteifio, J. O., Tibe, O. & Njogu, R. M. (2006). The mineral composition of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) grown in Southern Africa. *African Journal of Biotechnology*. 5 (23): 2408–2411, 2006.

Brink, M., Ramolemana, G. M. & Sibuga, K. P. (2006). *Vigna subterranea* (L.) Verdc. In Brink, M. and Belay, G. (Editors). *Plant Resources of Tropical African 1.Cereals and pulses.* PROTA Foundation, Wageningen, Netherlands. Pp. 213-218.

Linnemann, A. R. (1995). Phenological Development in Bambara groundnut (*Vigna subterranea*) transferred from 14 to 11 hour photoperiods. *Journal of Agricultural Science*. Cambridge Univ. Press. https://doi.org/10.1017/S0021859600073111

Mkandawire, C. H., (2007): Review of Bambara groundnut (*Vigna subterranean* (L.) Verdc.) Production in sub-Saharan Africa. *Agricultural Journal, 2*, 464-70.

Ndiang, Z., Bell, J. M., Fokam, P. E., Ouattara, B., Simo, C., & Dibong, D. S. (2014). Agro-morphological variability in twelve Bambara groundnut (*Vigna subterranea* (L.)Verdc.) accessions in Cameroon. *Sciences, Technologies & Développement,16*, 38-45.

Odongo, F. O., Oyoo, M. E., Wasike, V., Owuoche, J. O., Karanja, L. & Korir, P. (2015). Genetic diversity of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) landraces in Kenya using microsatellite markers. *African Journal of Biotechnology, 14*(4), 283-291. https://doi.org/10.5897/AJB2014.14082

Ouedraogo, M., Ouédraogo, J. T., Tignere, J. B., Balma, D., Dabire, C. B. & Konaté, G. (2008). Characterization and evaluation of accessions of Bambara groundnut [*Vigna subterranea* (L.) Verdcourt] from Burkina Faso. *The Science* *of Nature, 5*(2), 191-197. https://doi.org/10.4314/scinat.v5i2.42164

Rao, V. R., & Hodgkin, T. (2002). Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell Tissue Organ Culture, 68,* 1-19. https://doi.org/10.1023/A:1013359015812

Sevik, H., Ayan, S., Turna, I., & Yahyaoglu, Z. (2010). Genetic diversity among populations in Scotch pine (*Pinus sylvestris* L.) seed stands of Western Black Sea Region in Turkey. *African Journal of Biotechnology, 9*(43), 7266-7272.

Shegro, A. G., Jansen Van Rensburg, W. S., & Adebola, P. O. (2013). Assessment of genetic variability in Bambara groundnut (*Vigna subterranea* [L.] Verdc.) using morphological quantitative traits. *Academia Journal of Agricultural Research, 1,* 45-51.

doi:10.14720/aas.2020.116.1.1331 Review article / pregledni znanstveni članek

From plant biomass to biofuels and bio-based chemicals with microbial cell factories

Maša VODOVNIK [1, 2], Matevž ZLATNAR [1]

Received November 15, 2019; accepted July 20, 2020.
Delo je prispelo 15. november 2019, sprejeto 20. julij 2020.

From plant biomass to biofuels and bio-based chemicals with microbial cell factories

Abstract: Global energy demands and global warming represent key challenges of the future of human society. Continous renewable energy supply is key for sustainable economy development. Waste plant biomass represent abundant source of renewable energy that can be transformed to biofuels and other value-added products, which is currently limited due to the lack of cost-effective biocatalysts. The bottleneck of this process is the degradation of structural polysaccharides of plant cell walls to soluble compounds that can be fermented to solvents or transformed to biogas via methanogenesis and can be used as biofuels or chemical raw materials. In order to replace traditional physical and chemical methods of lignocellulose pretreatment with more environmentally friendly biological approaches, native microbial enzyme systems are increasingly being explored as potential biocatalysts that could be used in these processes. Microbial enzymes are useful either as catalysts in the enzymatic hydrolysis of lignocelluloses or as components incorporated in engineered microbes for consolidated bioprocessing of lignocelluloses. The unprecedented development of tools for genetic and metabolic engineering for a wide range of microorganisms enabled significant progress in the development of microbial cell factories optimized for the producton of biofuels. One of the most promising strategies aimed towards this goal, i.e. systematic design and heterologous expression of »designer cellulosomes« in industrial solventogenic strains is adressed in detail.

Key words: waste plant biomass; biocatalysts biofuels; solvents; microbial cell factories

Od rastlinske biomase do biogoriv in bio-surovin z mikrobnimi celičnimi tovarnami

Izvleček: Naraščajoče potrebe po obnovljivih virih energije in globalno segrevanje predstavljata ključna izziva prihodnosti človeške družbe. Za trajnostni razvoj industrije je nujna neprekinjena oskrba z obnovljivo energijo. Odpadna rastlinska biomasa predstavlja velik rezervoar obnovljive energije, ki jo je mogoče pretvoriti v biogoriva in druge produkte z dodano vrednostjo, vendar trenutno za tovrstno pridobivanje energentov še ni na voljo dovolj učinkovitih biokatalizatorjev. Omejujoča stopnja mikrobiološke pretvorbe odpadne rastlinske biomase v produkte z veliko dodano vrednostjo je hidroliza strukturnih polimerov do topnih podenot, ki predstavljajo substrat za fermentacijo do biogoriv in biosurovin z večjo dodano vrednostjo. V želji, da bi drage in neekološke tradicionalne postopke fizikalne in kemične predobdelave lignoceluloznih substratov nadomestili z okoljevarstveno sprejemljivejšimi biološkimi, v zadnjem desetletju poteka intenzivno proučevanju mikrobnih encimskih sistemov za razgradnjo rastlinske biomase. Vzporedno je nesluten razvoj orodij za gensko in metabolno spreminjanje mikrobnih celic omogočil pomembne dosežke na področju načrtovanja mikrobnih celičnih tovarn za optimizirano proizvodnjo biogoriv. Ena od obetavnejših naprednih strategij usmerjenih k temu cilju je tudi sistematično načrtovanje in ekspresija celulosomov »po meri« v solventogenih industrijskih sevih, čemur smo v pregledu posvetili največ pozornosti.

Ključne besede: odpadna rastlinska biomasa; biokatalizatorji, biogoriva; topila;mikrobne celične tovarne

---

[1] University of Ljubljana, Biotechnical Faculty, Chair of Microbiology and Microbial Biotechnology, Ljubljana, Slovenia
[2] Corresponding author, e-mail: masa.vodovnik@bf.uni-lj.si

1 INTRODUCTION

Global society is confronted with climate change, which is frequently associated with excessive consumption of fossil fuels, currently the most important source of energy on the planet. Moreover, the price of fossil fuels will continue to rise gradually as global demand grows and supply decreases (Shafiee and Topal, 2009). Some forcasts estimate consumption growth of up to 56 % between 2010 and 2040 (Azad et al., 2019). Consequently, new renewable and environmentally friendly energy resources are gaining in importance. One example of such bio-based energy resources are biofuels, which are based on microbial processes for transforming various types of biomass into energy-rich solvents or gases (Zhang et al., 2019). The first generation of biofuels was based on the use of easily degradable substrates (corn, grain, sugar cane, etc.). However, this strategy has ethical and ecological disadvantages, as it leads to the cultivation of monocultures. This could lead to an unacceptable reduction of agricultural land used for food production, with the potential to cause higher food prices and a reduction in biodiversity or even deforestation (Subramaniam et al., 2020, Hahn-Haegerdal et al., 2006). Therefore, it was soon recognized that biofuels should be produced from waste products instead of food crops. One such option is waste plant biomass, accumulating as a by-product of agriculture and food industry. The biggest bottleneck in the production of these, so-called second generation biofuels, is the conversion of the lignocellulosic biomass into soluble compounds that could easily be fermented to biofuels or other bio-based raw materials (chemicals and industrially-relevant enzymes). This step is enzymatically demanding and cannot be performed by most of the naturally solventogenic strains used in industrial processes. To facilitate the degradation of lignocellulose to fermentable compounds, pretreatment with concentrated chemicals (acids, bases, organic solvents or oxidants) and/or physical pretreatments (treatment with hot steam under pressure, pyrolysis, ultrasonic treatment) has traditionally been used. Such procedures are associated with environmental and cost burden (Chen et al., 2017). On the other hand, cost-efficiency of biological pretreatment processes is still too low for a wider industrial use.

To solve this issue, a considerable part of research is focused on the discovery and optimization of biocatalysts that would make this step economically viable for industrial production (Klein-Marcuschamer et al., 2011). Apart from their use for second generation biofuel production, optimized enzyme coctails could also improve the production of third-generation biofuels based on microalgal biomass (Venkata Mohan et al., 2016).

Over the last ten years, celullolytic systems have attracted a great deal of interest from the energetic industry. As many anaerobic cellulolytic microorganisms naturally convert (hemi)cellulose to ethanol they represent a promising alternative to conventional processes. However, the production yields in wild strains are not satisfactory for industrial applications.

In order to achieve the highest possible yields of biofuels, research is concentrating on the development of strains with improved properties. Various strategies are used for this purpose, in particular targeted evolution (random mutagenesis and screening for usable mutants) or targeted genetic manipulation of wild strains (Choi et al., 2020). A prerequisite for the second approach is the adequate characterization of restriction modification, transport and cellulolytic systems and metabolic pathways involved in the fermentation of sugars (Majidian et al., 2018). Exhaustive basic knowledge of native cellulolytic systems as well as potential production strains is essential for the development of strains with the desired properties and can be gained via genomic, transcriptomic and proteomic approaches as well as *in vitro* studies of interactions between individual enzymes. This review is focused on important scientific findings in the field of microbial enzyme systems for the degradation of lignocellulosic biomass and selected results of metabolic engineering experiments to design microbial cell factories for the production of second generation biofuels.

2 NATIVE MICROBIAL SYSTEMS FOR LIGNOCELLULOSIC BIOMASS DEGRADATION

The main component of accumulating plant biomass are the cell walls. This is associated with its structural recalcitrance, preventing most (micro)organisms to decompose this abundant energy source. The most abundant compund in the plant cell walls is cellulose, which typically accounts for 20 % to 90 % of the dry mass of plant tissues. This homopolysaccharide consists of 100 - 20 000 D-glucopyranose residues linked by 1-4 β-glycosidic bonds (O'Sullivan, 1997). Uniform structure and charge distribution in glucan chains enables the formation of multiple intramolecular hydrogen bonds, Van der Walls and hydrophobic interactions, These are responsible for tight packing of these molecules to microfibrils (30-36 glucan chains) and further to macrofibrils, rendering cellulose insoluble and inaccessible to enzymes (Keegstra, 2010). In most natural cellulose substrates, regions with a high order rate (crystalline regions) predominate and are partially interrupted by disordered (amorphous) regions. For an efficient decomposition of natural plant biomass the synergistic action of several enzymes with different specificities is necessary. Only some bacteria, fungi and very few protists contain this type of enzyme consortia. Two main types of cellulolytic systems that are found in nature are (1) free enzyme systems and (2) the cellulosomes. Free enzyme systems are typically found in aerobic cellulolytic microorganisms, which produce large amounts of free or membrane proteins that bind separately to the substrate. On the other hand, some anaerobes developed a more efficient strategy based on combining enzymes in complexes, namely cellulosomes (Vodovnik and Marinšek Logar, 2010). A limiting factor in the process of enzymatic decomposition of cellulose is poor access of glucan chains within highly ordered and strongly connected regions of microfibrils. Loosening of cellulose structure is therefore crucial first step of its degradation, as it makes a larger surface area available for hydrolytic and oxidative enzymes. This process is called amorphogenesis and is still being investigated. Cellulose binding modules (CBMs) of enzymes and cellulosomic structural proteins (scaffoldins) have been shown to play an important role, as well as specialized proteins called expansinsins. (Arantes in Saddler, 2010). After the cellulose network is loosened, hydrolytic enzymes (β-1,4-glucanases) can access individual cellulose chains and cleave them to soluble cellodextrins with a degree of polymerisation of 2-6. These are easily converted to glucose before or during the transfer across the cell wall and enter central fermentation pathways. Considering the mode of action and specificity toward the substrate, the cellulases can be classified as endoglucanases and exoglucanases (cellobiohydrolases). Endo-β-glucanases produce cello-oligosaccharides by randomly acting on the cellulosic chain within amorphous regions of cellulose. Released free ends represent the substrate for exoglucanases which release sugars from the end of the molecules. Cellulolytic microorganisms always produce both types of the cellulases that work synergistically (Moraïs et al., 2016). Traditionally, cellulases have been classified as glycoside hydrolases. Only recently, oxidative activity was detected in some of these enzymes. These enzymes were found in some aerobic microorganisms and were classified as lytic monooxygenases (Frandsen et al., 2016).

Cellulose fibrils in plant cell walls are incorporated in a matrix of hemicellulose and pectin, in addition to various glycoproteins and sometimes lignin. Consequently, the vast majority of cellulolytic microorganisms also produces enzymes necessary for the degradation of these molecules, particularly hemicellulases. One of the main components of the hemicellulose matrix in plant cell walls is xylan. This is branched molecule, with diverse substituents. Endoxylanases catalyze the hydrolysis of 1,4-β-glycosidic bonds within the main xylan backbone, releasing various oligoxylosaccharides, xylobiose and xylose. Exoxylanases cleave successive residues of D-xylose from the ends of the main xylan molecule. Finally, β-xylosidases hydrolise shorter oligoxylosaccharides (mostly xylobiose) to xylose (Malgas et al., 2019). These enzymes are present in most hemicellulolytic microorganisms and are usually cell-bound or released to the supernatant. The efficiency of xylanases depends on synergistic activity with auxiliary hemicellulytic enzymes (arabinofuranosidases, glucorunidases, esterases, etc.) which cleave side chains (arabinose, glucuronic acids, fenol and acetyl substituents) from the xylan backbone and separate it from lignin. In addition to hemicellulases, some aerobic microorganisms (mostly fungi) also produce ligninolytic oxidative enzymes, lacases and peroxidases (Plácido et al., 2015). In nature, decomposition of the complex matrix in plant cell walls is usually performed by coordinated action of different microorganisms, that either produce enzymes actively involved in the decomposition of polysaccharides or indirectly influence the degradation, e.g. by consuming end products to avoid catabolic repression. Such systems are too complex to be directly used for industrial applications, so different strategies are being used to develop better adapted, cost-efficient catalysts. Some major approaches will be discussed in the next chapters.

3 DEVELOPMENT OF CONSOLIDATED BIOPROCESSES

As mentioned above, one of the main strategies striving towards more efficient conversion of lignocellulose to biofuels is the development of consolidated bioprocesses. These are processes which alow direct (one-step) conversion of biomass to biofuel or other value-added compounds within a single system. Two approaches are commonly used to create such system. One option is to complement selected industrial solvent-producing strain with the genes encoding (ligno)cellulolytic enzymes. Alternatively, metabolic pathways of native cellulolytic strains can be manipulated towards increased production of the desired products. In both cases, the strains also need to be adapted to tolerate process conditions and high concentrations of produced solvents. This can be done by metabolic engineering or directed evolution coupled to strain selection (Olson et al., 2012).

Apart from strain development by metabolic engineeging, consolidated bioprocesses can also take place in stable mixed culture (co-culture), composed of two or more different strains or species specialized for different process stages (Yang et al., 2015). Argyros et al. (2011), for example, developed a co-culture system with the cellulolytic bacteria *Clostridium thermocellum* and the solvent-producing *Thermoanaerobacter saccharolyticum* and achieved about 80 % of the theoretical maximum ethanol yield from microcrystalline cellulose (Avicel substrate). A co-culture system of *C. thermocellum* combined with *C. thermohydrosulfuricum* or *Thermoanaerobacter pseudoethanolicus* growing on cellulose substrate has also been reported to achieve better ethanol conversion than *C. thermocellum* as a monoculture (Levin et al., 2015). One of the main advantages or even the main benefit of the mixed culture system is the reduction of catabolic suppression of enzymes when less recalcitrant carbon sources are also present in the substrates(Liu et al., 2019).

4 MICROBIAL CELL FACTORIES FOR THE PRODUCTION OF BIOFUELS

Microbial cell factory design is an approach to bioengineering which considers microbial cells as a production facility in which the optimization process largely depends on metabolic engineering. Microbial cell factories are used for many applications in industrial microbiology and also hold promise for bioenergetics. With the intention of developing industrial strains for cost-efficient conversion of lignocellulosic waste to biofuels and other value-added products, research focuses on the adaptation of selected strains with addition, silencing, modification or overexpression of different genes involved in target metabolic pathways (Das et al., 2020). Among the strategies used for metabolic engineering to enhance biofuel production and facilitate utilization of non-edible low-value carbon sources are carbon flux rerouting, reducing power enhancement, enzyme engineering, pathway design/discovery, overcoming product toxicity and protein secretion optimization were already applied (Choi et al., 2020).

The biosynthesis of lignocellulolytic enzymes in micro-organisms is regulated by transcription factors that are activated in the presence of lignocellulosic materials (Aro et al., 2005). Genes encoding these proteins thus represent a potential target for increasing the production of targeted enzymes through genetic modification. Su et al (2009) created artificial imitations of transcription factors involved in the regulation of lignocellulolytic glycoside hydrolases and achieved a higher production these enzymes in strains with artificial regulation.

To facilitate the utilization of lignocellulosic derivatives, heterologous machineries for catabolizing xylose, arabinose, cellulose and lignin have also been screened and introduced to the production hosts. Furthermore, engineering of protein folding and post-translational modification systems to enhance overexpression of the heterologous enzymes, genome scale modelling and ALE to overcome catabolite repression have so far been effective in enhancing utilization of lignocellulosic derivatives (Choi et al., 2020).

Furthermore, a very promising strategy to improve the yield of target enzymes focuses on the regulation of their transport and secretion from cells (Idiris et al. 2010; Reed and Chen, 2013). Pakula et al (2005) investigated the secretome of the hemicellulase producing fungus *Trichoderma reesei* Simons and noticed that only a small fraction of produced enzymes is actually secreted out of the cells, which confirmed that secretion is one of the bottlenecks in protein production and can be targeted for improvement. Furthermore, recombinant enzymes are useful either as catalysts in the enzymatic hydrolysis of lignocelluloses or as components incorporated in engineered microbes for consolidated bioprocessing of lignocelluloses. If microbial cells expressing recombinant proteins are used to interact with polymeric material, such as lignocelluloses, extracellular secretion of proteins is necessary due to the inability of microbial cells to uptake polymer substrates. In the case of applications where purified recombinant proteins are used directly, secretion of these proteins extracellularly could significantly reduce the complexity of a production process by eliminating the need for cell lysis and reducing the burden of removing host proteins. In addition, secretion of highly expressed proteins minimizes formation of inclusion bodies due to environment better suited for folding and disulfide bond formation, reducing the effects of intracellular protein degradation and lessening the detrimental effects of cytotoxic proteins (Reed and Chen, 2013; Burdette et al., 2018). The signal peptide sequence is one of the most important factors influencing secretion efficieny. A common approach is to fuse an optimized signal sequence of the expression host to the target protein. Such endogenous signal peptide fusion approach has for example been used in engineering *B. subtilis* (Ehrenberg 1835) Cohn 1872 secreting *Clostridium cellulovorans* Sleat et al. 1985 mini-cellulosome (Arai et al., 2007). Signal peptides from microorganisms other than expression host may also be used (exogenous peptides). As with the endogenous pepides, selection of exogenous signal sequences often entail screening a large library of candidate signal sequences (Reed & Chen, 2013).

Recent research has shown that the efficiency of cellulose degradation depends more on the degree of synergy between different enzymes than on the absolute activity of individual enzymes. This knowledge led to the development of designer cellulosomes – artificially constructed enzyme complexes tailored for optimized degradation of different lignocellulose substrates. In these recombinant cellulosomes, chimeric subunits from different microorganisms are combined for increased efficiency of target substrate degradation (Figure 1). Studies with designer minicellulosomes, incorporating different enzyme combinations allow the identification of catalytic subunit sets with superb activity (Leis et al., 2018). At present, designed minicellulosomes are mainly used for interaction studies, but increasing number of experiments performed suggests a possible future industrial application. The genes encoding cellulosomes with the desired subunits have already been transfered to selected microorganisms without natural ability to degrade cellulose, which are able to produce a wide range of useful chemicals as fermentation products (ethanol, buthanol, acetone, etc.) (Lütke-Eversloh, 2014, Willson et al., 2016). In recent research Moraïs et al. (2016) constructed a minicellulosome combining exoglucanase Cel48S, endoglucanase Cel8A and β-glucosidase BglA from the bacterium *Clostridium termocellum* NCBI, that have previously been improved by targeted and random mutagenesis. The complexes demonstrated improved stability and activity at high temperatures than original cellulosomes, which is particularly promising for application in thermophilic industrial processes.

The expression of minicellulosomes in industrial strains of fungi, has also been reported. *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen, for example, has been used for thousands of years for the production of ethanol. It is one of the most important industrial microorganisms, not only due to its efficient production of ethanol, but also due to its rapid growth, flexible metabolism, ability to grow in anaerobic environments and tolerance for higher concentrations of ethanol than most of bacteria (Nevoigt, 2008). However, this yeast does not have the ability to degrade plant cell walls (Piškur in Langkjaer, 2004). Tsai et al (2009) reported succesfull expression of a minicellulosome combining enzymes from two different *Clostridia* and *Ruminococcus* within *S. cerevisie*. The recombinant strain was capable of simultaneous cellulose saccharification and fermentation of the produced sugars, which makes it interesting for industrial production of bioethanol.

Apart from ethanol, buthanol is also considered a promising biofuel. Moreover, compared to ethanol, buthanol is more energy-dense, less corrosive and less volatile, which makes it even more appealing for industrial applications (da Silva Trinidade and dos Santos, 2017). Buthanol and some other higher alcohols are also more compatible with current infrastructure and mechanisation (Kótai et al., 2013). The most important current producer of biobuthanol is the thermophilic bacterium *Clostridium acetobutylium* Mccoy et al. 1926 emend. Keis et al. 2001, which is known for its bi-phasic fermentation: acidogenic and solventogenic. In acidogenic phase the cells convert substrate into acetic and butyric acid, while in the solventogenic phase they convert it into acetone, ethanol and buthanol in a ratio of 3:6:1. This phase is also known as ABE fermentation (Xue et al., 2017). Native species, however, can only ferment sugars to usable solvents and are therefore not suitable for consolidated bioprocesses leading to the production of second-generation biofuels. To overcome this obstacle, Willson et al (2016) inserted synthetic gene constructs encoding cellulosomic structural and enzyme subunits from the related cellulolytic *C. cellulolyticum* to the native *C. acetobutylicum* strain. One of the main challenges limiting the industrial production of biobuthanol is the toxicity of this solvent to most of the producing strains. Consequently, the production is stopped at lower concentrations than in case of bioethanol, leading to higher purification costs. Most of the efforts are therefore focused

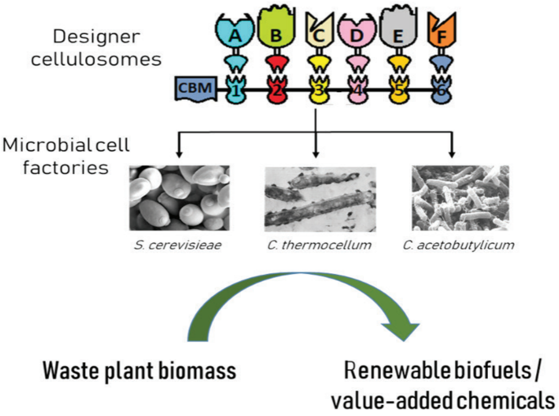


Figure 1: Microbial cell factories for production of second generation biofuels: heterologous expression of designer cellulosomes in industrial solventogenic strains (authors of SEM photographies: *C. thermocellum*: E. Bayer and R. Lamed, *S. cerevisiae*: Das Murtey and Ramasamy, 2016, *C. acetobutylicum*: BacMap Genome Atlas).

on developing more robust strains with higher tolerance to buthanol and ABE fermentation by-products. For this purpose, metabolic engineering approaches and random muthagenesis have already been used (Xue et al., 2017). Another strategy to increse the cost-efficiency of the processes in second-generation biofuel production is also to diminish product purification costs by reducing the formation of by-products. However, this approach has so far proved challenging. Tummala et al (2003) attempted to regulate solvent production in *Clostridium acetobutylicum* by switching off the gene for acetoacetate decarboxylase (*adc*) by small antisense RNA molecules, but were unsuccesful. However, the resutls of the study revealed that CoAT is the rate-limiting enzyme in acetone formation process. Furthermore, Jiang et al. (2009) completely disrupted *adc* gene with the insertion of a plasmid, which led to increased buthanol production (up to 70 % of fermentation products). Nevertheless, industrial strains for biobuthanol production are currently still far from being economically viable. It is clear that many improvements still need to be performed. A promising approach that may solve some current challenges associated with genetic modification of solventogenic *Clostridia* involves use of CRISPR-Cas system, which has already been used to improve some related *species* (Xue et al., 2017; Das et al. 2020).

5 IMPROVEMENT THE BIOMASS DECOMPOSITION PROPERTIES

One of the approaches aimed for optimizing the production of biofuels from plant biomass is also genetic modification of the plants. Huang et al (2019), for example, created transgenic rice plants by overexpresseing two glycoside hydrolases involved in modification of cellulose-containing microfibrils. The plant cell wall of modified plants had lowered crystallinity index and cellulose depolarisation factor, which resulted in higher digestibility and increased bioethanol yields.

Many similar experiments to modify the biomass by regulating the expression of intrinsic plant (hemi)cellulases were carried out, but are always challenged by the side effects, particularly deterioration in the mechanical stability and crop yield. In addition, due to strict regulation associated with the use of genetic manipulation of plants currently limits the use of these plants for the general production of second-generation biofuels from waste plant biomass (Joshi in Nookaraju, 2012).

6 CONCLUSION

In December 2018, the revised renewable energy directive 2018/2001/EU entered into force, as part of the Clean energy for all Europeans package, aimed at keeping the EU a global leader in renewables and, more broadly, helping the EU to meet its emissions reduction commitments under the Paris Agreement. The new directive establishes a new binding renewable energy target for the EU for 2030 of at least 32 %, with a clause for a possible upwards revision by 2023. The directive also encourages permament development for EU to change the direction of bioenergy towards greater savings in greenhouse gas emissions and the reduction of undesirable effects on the environment, particularly in the case of indirect changes in the use of agricultural land for the production of biofuels from crops despite the use of this land as food or feed. The development of more efficient biocatalysts and systems that will allow for the economically viable production of sustainable energy from plant biomass is certainly one of the promising approaches toward these goals. New discoveries resulting from intensive research in the field of microbial systems for the degradation of plant biomass by applying system's biology approaches combined with new tools for the genetic modification of microbial cells (including CRISPR-Cas system) will certainly play a crucial role towards optimization of individual enzymes as well as the entire microbial cells.

7 REFERENCES

Arantes, V., Saddler, J.N. (2010). Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. *Biotechnology for Biofuels, 3*(4),. https://doi.org/10.1186/1754-6834-3-4

Arai, T., Matsuoka, S., Cho, H.-Y., Yukawa, H., Inui, M., Wong, S.-L., Doi, R.H. (2007). Synthesis of Clostridium cellulovorans minicellulosomes by intercellular complementation. *Proceedings of National Academy Of Science, 104*, 1456–1460. https://doi.org/10.1073/PNAS.0610740104

Argyros, D.A., Tripathi, S.A., Barrett, T.F., Rogers, S.R., Feinberg, L.F., Olson, D.G., ..., Hogsett, D.A. (2011). High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. *Applied and Environmental Microbiology, 77*(23), 8288–8294. https://doi.org/10.1128/AEM.00646-1

Aro, N., Pakula, T., Penttila, M. (2005). Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiology Reviews, 29*(4), 719-739. https://doi.org/10.1016/j.femsre.2004.11.006

Azad, K., Rasul, M.G., Khan, M.M.K., Sharma, S.C. (2019). Introduction to sustainable and alternative ecofuels, in: Azad, K. (Ed.), *Advances in Eco-Fuels for a Sustainable Environment*, Woodhead Publishing Series in Energy. Woodhead Publishing, pp. 1–14. https://doi.org/https://doi.org/10.1016/B978-0-08-102728-8.00001-2

Burdette, L.A., Leach, S.A., Wong, H.T., Tullman-Ercek, D. (2018). Developing Gram-negative bacteria for the secretion of heterologous proteins. *Microbial cell factories*, *17*. https://doi.org/10.1186/s12934-018-1041-5

Chen, H., Liu, J., Chang, X., Chen, D., Xue, Y., Liu, P., Lin, H., Han, S. (2017). A review on the pretreatment of lignocellulose for high-value chemicals. *Fuel processing Technology, 160*, 196–206. https://doi.org/https://doi.org/10.1016/j.fuproc.2016.12.007

Choi, K.R., Jiao S., Lee, S.Y. (2020). Metabolic engineering strategies toward production of biofuels. *Current opinion in Chemical Biology, 59*, 1–14. https://doi.org/https://doi.org/10.1016/j.cbpa.2020.02.009

Conti, J.J. (2011). *International Energy Outlook*. IEA: 246 str. https://www.eia.gov/outlooks/aeo/pdf/03832011.pdf (23.8.2019)

EU. (2018/2001). Directive (eu) 2018/2001 of the european parliament and of the council. Retrieved from https://eur-lex.europa.eu/eli/dir/2018/2001/oj

Hahn–Hägerdal, B., Galbe, M., Gorwa–Grauslund, M.F., Liden, G., Zacchi. G. (2006). Bio– ethanol: the fuel of tomorrow from the residues of today. *Trends in Biotechnology, 24*(12), 549–556. https://doi.org/10.1016/j.tibtech.2006.10.004

Huang, J., Xia, T., Li, G., Li, X., Li, Y., Wang, Y., … Wang, L. (2019). Overproduction of native endo-β-1,4-glucanases leads to largely enhanced biomass saccharification and bioethanol production by specific modification of cellulose features in transgenic rice. *Biotechnology for Biofuels, 12*(11). https://doi.org/10.1186/s13068-018-1351-1

Idiris, A., Tohda, H., Kumagai, H., Takegawa, K. (2010). Engineering of protein secretion in yeast: strategies and impact on protein production. *Applied Microbiology and Biotechnology, 86*(2), 403–417. https://doi.org/10.1007/s00253-010-2447-0

Jiang, Y., Xu, C., Dong, F., Yang, Y., Jiang, W., Yang, S. (2009). Disruption of the acetoacetate decarboxylase gene in solvent–producing Clostridium acetobutylicum increases the butanol ratio. *Metabolic Engineering, 11*(4–5), 284–291. https://doi.org/10.1016/j.ymben.2009.06.002

Joshi, C.P., Nookaraju, A. (2012). New Avenues of Bioenergy Production from Plants: Green Alternatives to Petroleum. *Journal of Phylogenetics and Evolutionary Biology, 3*, 134. https://doi.org/10.4172/2157-7463.1000134

Keegstra, K. (2010). Plant cell walls. *Plant Physiology, 154*, 483–486. https://doi.org/10.1104/pp.110.161240

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B.A., Blanch, H.W. (2011). The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnology and Bioengineering, 109*(4), 1083-1087. https://doi.org/10.1002/bit.24370

Kótai, L., Szépvölgyi, J., Szilágyi, M., Zhibin, L., Baiquan, C., Sharma, V., Sharma, P.K. (2013). Biobutanol from renewable agricultural and lignocellulose resources and its perspectives as alternative of liquid fuels. In Z. Fang (ed.) *Liquid, gaseous and solid biofuels– conversion techniques* (pp. 199-262). Rijeka, InTech. https://doi.org/10.5772/52379

Frandsen, K.E.H., Simmons, T.J., Dupree, P., Poulsen, J.C.N., Hemsworth, G.R., Ciano, L., ... Walton, P.H. (2016). The molecular basis of polysaccharide cleavage by lytic polysaccharide monooxygenases. *Nature Chemical Biology, 12*, 298-303. https://doi.org/10.1038/nchembio.2029

Leis, B., Held, C., Andreeßen, B., Liebl, W., Graubner, S., Schulte, P., Schwarz, W., Zverlov, V. (2018). Optimizing the composition of a synthetic cellulosome complex for the hydrolysis of softwood pulp: Identification of the enzymatic core functions and biochemical complex characterization. *Biotechnology for Biofuels, 11*. https://doi.org/10.1186/s13068-018-1220-y

Levin, D., Verbeke, T., Munir, R., Islam, R., Ramachandran, U., Lal, S., Schellenberg, J., Sparling, R. (2015). Omics approaches for designing biofuel producing cocultures for enhanced microbial conversion of lignocellulosic substrates. In: M.E. Himmel, M.E. (Ed.), *Direct Microbial Conversion of Biomass to Advanced Biofuels* (pp. 335-363). Elsevier. https://doi.org/10.1016/B978-0-444-59592-8.00017-8

Liu, J., Shi, P., Ahmad, S., Yin, C., Liu, X., Liu, Y., Zhang, H., Xu, Q., Yan, H., & Li, Q. (2019). Co-culture of Bacillus coagulans and Candida utilis efficiently treats *Lactobacillus* fermentation wastewater. *AMB Express*, *9*(1), 15. https://doi.org/10.1186/s13568-019-0743-3

Lütke-Eversloh, T. (2014). Application of new metabolic engineering tools for *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology, 98*(13), 5823-5837. https://doi.org/10.1007/s00253-014-5785-5.

Majidian, P., Tabatabaei, M., Zeinolabedini, M., Naghshbandi, M. P., Chisti, Y. (2018). Metabolic engineering of microorganisms for biofuel production. *Renewable and Sustainable Energy Reviews, 82*(3), 3863–3885. https://doi.org/10.1016/j.rser.2017.10.085

Malgas, S., Pletschke, B.I.(2019). The effect of an oligosaccharide reducing-end xylanase, BhRex8A, on the synergistic degradation of xylan backbones by an optimised xylanolytic enzyme cocktail. *Enzyme Microbial Technology, 122*, 74–81. https://doi.org/10.1016/j.enzmictec.2018.12.010

Moraïs, S., Stern, J., Kahn, A., Galanopoulou, A.P., Yoav, S., Shamshoum, ...Bayer, E. A. (2016). Enhancement of cellulosome-mediated deconstruction of cellulose by improving enzyme thermostability. *Biotechnology for Biofuels, 9*(164). https://doi.org/10.1186/s13068-016-0577-z

Das, M., Patra, P., Ghosh, A. (2020). Metabolic engineering for enhancing microbial biosynthesis of advanced biofuels. *Renewable and Sustainable Energy Reviews, 119*, 109562. https://doi.org/https://doi.org/10.1016/j.rser.2019.109562

Das Murtey, M.,&6 Ramasamy, P. (2016). Sample preparations for scanning electron microscopy – Life Sciences. In: M. Janicek & R. Kral (Eds.), *Modern Electron Microscopy in Physical and Life Sciences*, + IntechOpen. https://doi.org/10.5772/61720

Nevoigt E. (2008). Progress in metabolic engineering of Saccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews, 72*(3), 379–412. https://doi.org/10.1128/MMBR.00025-07

Olson, D.G., McBride J.E., Shaw, A.J., Lynd, L.R. (2012). Recent progress in consolidated bioprocessing. *Current Opinion in Biotechnology, 23*(3), 396–405. https://doi.org/10.1016/j.copbio.2011.11.026

O'Sullivan, A.C. (1997). Cellulose: the structure slowly unravels. *Cellulose, 4*, 173–207. https://doi.org/10.1023/A:1018431705579

Pakula, T.M., Salonen K., Uusitalo, J., Penttila, M. (2005). The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus *Trichoderma reesei*. *Microbiology, 151*(1), 135-143. https://doi.org/10.1099/mic.0.27458-0

Piškur, J., Langkjaer, R.B. (2004). Yeast genome sequencing: the power of comparative genomics. *Molecular Microbiology, 53*(2), 381–389. https://doi.org/10.1111/j.1365-2958.2004.04182.x

Plácido, J., Capareda, S. (2015). Ligninolytic enzymes: a biotechnological alternative for bioethanol production. *Bioresources and Bioprocesses, 2*(23). https://doi.org/10.1186/s40643-015-0049-5

Reed, B., Chen, R. (2013). Biotechnological applications of bacterial protein secretion: from therapeutics to biofuel production. *Research in Microbiology, 164*, 675–682. https://doi.org/https://doi.org/10.1016/j.resmic.2013.03.006

Shafiee, S., Topal, E. (2009). When will fossill fuel reserves be diminished? *Energy Policy, 37*(1), 181–189. https://doi.org/10.1016/j.enpol.2008.08.016

da Silva Trindade, W.R., dos Santos, R.G. (2017). Review on the characteristics of butanol, its production and use as fuel in in ternal combustion engines. *Renewable & Sustainable Energy Reviews, 69*, 642–651. https://doi.org/10.1016/j.rser.2016.11.213

Su, X., Chu, X., Dong, Z. (2009). Identification of elevated transcripts in a *Trichoderma reesei* strain expressing a chimeric transcription activator using suppression subtractive hybridization. *World Journal of Microbiology and Biotechnology, 25*(6), 1075-1084. https://doi.org/10.1007/s11274-009-9993-6

Subramaniam, Y., Masron, T.A., Azman, N.H.N. (2020). Biofuels, environmental sustainability, and food security: A review of 51 countries. *Energy Research and Social Science, 68*, 101549. https://doi.org/https://doi.org/10.1016/j.erss.2020.101549

Tsai, S.L., Oh, J., Singh, S., Chen, R., Chen, W. (2009). Functional assembly of minicellulosomes on the *Saccharomyces cerevisiae* cell surface for cellulose hydrolysis and ethanol production. *Applied and Environmental Microbiology, 75*(19), 6087–6093. https://doi.org/10.1128/AEM.01538-09

Tummala, S.B., Welker, N.E., Papoutsakis, E.T. (2003). Design of antisense RNA constructs for downregulation of the acetone formation pathway of Clostrdidium acetobutylicum. *Journal of Bacteriology, 185*(6), 1923–1934. https://doi.org/10.1128/JB.185.6.1923-1934.2003

Venkata Mohan, S., Modestra, J.A., Amulya, K., Butti, S.K., Velvizhi, G. (2016). A circular bioeconomy with biobased products from CO2 sequestration. *Trends in Biotechnology, 34*, 506–519. https://doi.org/10.1016/j.tibtech.2016.02.012

Vodovnik, M., Marinšek-Logar, R. (2010). Cellulosomes – promising supramolecular machines of anaerobic cellulolytic-microorganisms. *Acta chimica slovenica, 57*, 767-774.

Willson, B.J., Kovács, K., Wilding-Steele, T., Markus, R., Winzer, K., Minton, N.P. (2016). Production of a functional cell wall-anchored minicellulosome by recombinant *Clostridium acetobutylicum* ATCC 824. *Biotechnology for Biofuels, 9*(109). https://doi.org/10.1186/s13068-016-0526-x

Xue, C., Zhao, J., Chen, L., Yang, S.T., Bai, F. (2017). Recent advances and state–of–the–art strategies in strain and process engineering for biobutanol production by *Clostridium acetobutylicum*. *Biotechnology Advances, 35*(2), 310–322. https://doi.org/10.1016/j.biotechadv.2017.01.007

Yang, X., Xu, M., Yang, S.T. (2015). Metabolic and process engineering of *Clostridium cellulovorans* for biofuel production from cellulose. *Metabolic Engineering, 32*, 39–48. https://doi.org/10.1016/j.ymben.2015.09.001

Zhang, J., Zhang, X. (2019). The thermochemical conversion of biomass into biofuels.In Verma D., Fortunati E., Jain S., Zhang X. (Eds.) *Biomass, biopolymer-based materials and Bioenergy* (pp. 327–368). Woodhead Publishing. https://doi.org/10.1016/B978-0-08-102426-3.00015-1

doi:10.14720/aas.2020.116.1.1593 Original research article / izvirni znanstveni članek

Investigation of gene expression diversity in *Hypericum* spp. before and after flowering under different nitrogen fertilization levels

Mahmood YASAMAN[1], Hossein ABBASPOUR[1, 2], Maryam PEYVANDI[1], Mohammad Reza NAGHAVI[3]

Received April 06, 2020; accepted June 22, 2020.
Delo je prispelo 06. aprila 2020, sprejeto 22. junija 2020.

Investigation of gene expression diversity in *Hypericum* spp. before and after flowering under different nitrogen fertilization levels

Abstract: The traditional medicinal herb, *Hypericum perforatum* L. has been popular for its pharmaceutical and coloring wealth since the ancient era. A secondary metabolite from the group of naphthodianthrones in *Hypericum* spp. named hypericin is responsible for the antidepression, anticancer, and antiviral characteristics of this herb. It has been found that several genes are involved in the biosynthesis pathway of hypericin. The *hyp-1* gene is participating in this biosynthesis path through the conversion of emodin to hypericin. The naphthodianthrones (hypericin and pseudohypericin) in *Hypericum* are synthesized through the polyketide pathway. In the plants, the enzyme complexes named polyketide synthase (PKS) catalyzes the reactions of polyketide pathways. The genes *HpPKS1* and *HpPKS2* are encoding PKS enzyme complexes. In this research, the relative expression of *hyp-1*, *HpPKS1,* and *HpPKS2* genes was compared in root and leaves of *Hypericum perforatum* and *H. androsaemum* L., before and after flowering under urea fertilization at 24, 48 and 72 hours after irrigation. The highest expression level of all three genes was observed after flowering in the samples of *H. perforatum* that were fertilized 72 hours after irrigation by 1 g l^{-1} urea (*hyp-1* in roots; *HpPKS1* and *HpPKS2* in leaves). The relative expression of *hyp-1* in the root was greater than in the leaves, but *HpPKS1* and *HpPKS2* expression in leaves was higher than in root. The relative expression of all three genes in *H. perforatum* was higher than in *H. androsaemum*. By increasing the interval between urea fertilization and irrigation, the relative expression of genes had an increasing trend, also by increasing the amount of urea fertilizer, relative gene expression was increased.

Key words: *Hypericum;* hypericin; *hyp-1 HpPKS1*; *HpPKS2*; transcriptional diversity

Preučevanje raznolikosti izražanja genov pri dveh vrstah krčnic (*Hypericum* spp.) pred in po cvetenju v razmerah različnega gnojenja z dušikom

Izvleček: Šentjanževka (*Hypericum perforatum* L.) je že od nekdaj popularna kot tradicionalno zdravilno zelišče zaradi svojih farmacevtskih in barvilnih lastnosti. Sekundarni metabolit iz skupine naftodiantronov v vrstah iz rodu *Hypericum* imenovan hipericin je odgovoren za antidepresivne, antikancerogene in antivirusne lastnosti tega zelišča. Ugotovljeno je bilo, da so pri biosintezi hipericina udeleženi številni geni. Gen *hyp-1* sodeluje pri tej biosintezi preko pretvorbe emodina v hipericin. Naftodiantrona (hipericin in pseudohipericin) se v šentjanževki sintetizirata po poliketidni poti presnove. V rastlinah katalizira reakcije poliketidne presnovne poti encimski kompleks poliketid sintaza (PKS). Gena *HpPKS1* in *HpPKS2* kodirata PKS encimski kompleks. V tej raziskavi je bila primerjana relativna ekspresija genov *hyp-1*, *HpPKS1,* in *HpPKS2* v koreninah in listih vrst *Hypericum perforatum* in *H. androsaemum* L., pred in po cvetenju, pri gnojenju z ureo 24, 48 in 72 ur po zalivanju. Največja ekspresija vseh treh genov je bila opažena po cvetenju v vzorcih vrste *H. perforatum*, 72 ur po tem, ko je bila rastlina fertirigirana z 1 g l^{-1} uree (*hyp-1* v koreninah; *HpPKS1* in *HpPK* (v listih). Relativna ekspresija *hyp-1* v koreninah je bila večja kot v listih, a ekspresija genov *HpPKS1* in *HpPKS2* v listih je bila večja kot v koreninah. Relativna ekspresija vseh treh genov je bila v vrsti *H. perforatum* večja kot v vrsti *H. androsaemum*. S povečevanjem intervala med gnojenjem z ureo in zalivanjem se je pokazal trend naraščajoče ekspresije genov, kar se je pokazalo tudi s povečevanjem odmerka uree.

Ključne besede: *Hypericum*; hipericin; *hyp-1 HpPKS1*; *HpPKS2*; transkripcijska raznolikost

---

[1] Islamic Azad University, Faculty of Biologica Sciences, North Tehran Branch, Department of Biology, Tehran, Iran
[2] Corresponding author, e-mail: abbaspour75@yahoo.com
[3] University of Tehran, Agricultural & Natural Resources College, Agronomy and Plant Breeding Department, Karaj, Iran

Abbreviations: AF: Sampling after flowering; BF: Sampling before flowering; 0: Non-fertilized; 0.5-24: 0.5 g l^{-1} urea fertilizer was used 24 hours after irrigation; 1-48: 1 g l^{-1} urea fertilizer was used 48 hours after irrigation. [Hp]: *Hypericum perforatum*; [Ha]: *Hypericum androsaemum*. cDNA: complementary DNA; DNA: Deoxyribonucleic acid; ds/m: deciSiemens per meter; g l^{-1}: grams per liter; *HpPKS1*: Polyketide synthase I; *HpPKS2*: Polyketide synthase II; ppm: parts per million; RNA: Ribonucleic acid; rRNA: Ribosomal ribonucleic acid; SAS: Statistical Analysis System

Okrajšave: AF: vzorčenje po cvetenju; BF: vzročenje pred cvetenjem; 0: negnojeno; pognojeno s 0,5-24: 0,5 g l^{-1} uree 24 ur po zalivanju; 1-48:pognojeno z 1 g l^{-1} uree 48 ur po zalivanju. [Hp]: *Hypericum perforatum*; [Ha]: *Hypericum androsaemum*. cDNA: komplementarna DNK; DNA: Deoksiribonukleinska kislina; ds m^{-1}: deci Siemens na meter; g l^{-1}: grami nar liter; *HpPKS1*: Poliketidna sintaza I; *HpPKS2*: Poliketidna sintaza II; ppm: delci na miljon; RNA: Ribonukleinska kislina; rRNA: Ribosomalna ribonukleinska kislina; SAS: Sistem statistične analize

1 INTRODUCTION

Medicinal plants are one of the most important potential sources of drugs that have been used since ancient times. The World Health Organization estimates that more than 80 percent of people use medicinal herbs through traditional or modern ways. Also, many chemical drugs are also made by modeling plant compounds (Samuelsson & Bohlin, 2017). In recent years, the increasing tendency to use medicinal herbs and their compounds have been observed, and currently, more than 40 percent of the drugs used in the advanced western countries are herbaceous origin (Khosh-Khui et al., 1984). Identifying and investigating the chemical compounds of medicinal plants, not only helps to treat diseases more easily and cheaply, but also leads to a decline in drug imports. Iran, with its special climatic conditions, has a vast abundance of local medicinal herbs and is a source of rich potential for these plants.

The genus *Hypericum* (Hypericaceae family) consists of 496 worldwide species (Nürk et al., 2013; Ruhfel et al., 2013), of which 17 species have been reported from Iran and most of them have antioxidants (Silva et al., 2005), antiviral (Birt et al., 2009), an antidepressant (Butterweck, 2003) and anticancer compounds (Agostinis et al., 2002). Most of these medicinal properties are due to the presence of a group of hydrosoluble secondary metabolites, which are often present in the flowers of this perennial herb (Lazzara et al., 2015). *Hypericum perforatum* L. is one of the traditional medicinal herbs that has been used since ancient times because of its secondary metabolites and coloring properties. *Hypericum* is one of the most important sources of dye in dyeing industries and is used for coloring silk and wool in red. The boiling of the vegetative body of *Hypericum* with greenery leads to the formation of yellow color for the dyeing industry (Duke, 2002; Russo et al., 2014).

Hypericin is the key biochemical compound used to determine the quality of the extract of *Hypericum* (Duppong et al., 2004), and it has been shown that the number of dark glands in the leaf is correlated with hypericin content (Briskin & Gawienowski, 2001). Hypericin is probably the strongest natural light-sensitive substance (photosensitizer) that has been identified so far, the photosensitizers are used in photodynamic therapy of cancers (Agostinis at al., 2002; Karioti & Bilia, 2010). Although the function of hypericin in *Hypericum* species is not yet fully understood, it has been suggested that this compound plays a defensive role against herbivorous organisms and plant pathogens (Sirvent et al., 2003; Bruni & Sacchetti, 2009). Through a chemical defense system, the phytophagous insects feeding on *H. perforatum* leaves produced more antioxidant enzymes under oxidative stress caused by hypericin photosensitization. Hypericin in sunlight interact with oxygen molecules to produce oxygen free radicals and create oxidative stress, which causes phytophagous insects' behavioral avoidance feeding on the plant tissue (Guillet et al., 2000). To determine the possible role of hypericin in the defensive response of *H. perforatum*, the effects of elicitors such as salicylic acid (SA) and methyl jasmonate (MeJA) on the production of secondary metabolites in *H. perforatum* were evaluated, which resulted in increased levels of hypericin in certain concentrations of SA and MeJA (Sirvent & Gibson, 2002). Hypericin provides the energy and health for human consumers and considerably enhances the human body's defensive ability through increasing cellular oxygen absorption and vascular respiration (Miskovsky, 2002: Agostinis at al., 2002). Regarding the photodynamic properties of hypericin, this metabolite has therapeutic potential as an antiviral, antiretroviral, and anticancer agent (Zhang et al., 2016, Jendželovská et al., 2016; Kubin et al., 2005). Hypericin, a valuable polycyclic dianthroquinone, is responsible for the antidepression and antiviral characteristics (Rahnavrd, 2017).

Studies have shown that the *hyp-1* gene is one of the most important genes involved in the pathway of hypericin biosynthesis, which encodes for phenolic coupling protein, that is catalyzed *in vitro* and specifically convert emodin to hypericin (Bais et al., 2003; Zhang et al., 2018; Michalska et al., 2010). The production of emodin anthrone, an anthraquinone which is a precursor for hypericins biosynthesis (Falk, 1999), proceeds through a polyketide pathway that is catalyzed by *HpPKS1* and *Hp-*

PKS2 (Zobayed et al., 2006; Michalska et al., 2010). Recently, it was proposed that probably the function of the Hyp-1 protein is more likely associated with the reserving or carrying of hypericin in the cell than in the real biosynthesis of hypericin (Michalska et al., 2010).

In detail, the octa-β-ketoacyl chain is made from acetyl-CoA plus malonyl-Coa that catalyzed by polyketide synthase (PKS). After several steps emodin is made, phenolic oxidative coupling protein (Hyp-1) catalyzes the emodin anthrone production from emodin, then Hyp-1 catalyzes the emodin anthrone conversion to protohypericin, and finally, Hyp-1 catalyzes the formation of hypericin from protohypericin. Therefore the genes *hyp-1*, *HpPKS1,* and *HpPKS2* are playing important roles in hypericin biosynthesis (Rahnavrd, 2017).

Through enhancing some components of a plant, such as increasing the plant secondary metabolites producing parts consequently will rise the accumulation of active ingredients and bioactive compounds (Jabbari et al., 2011). Some environmental factors like nitrogen fertilization play an important role in improving secondary metabolites content. Some nutrients (nitrogen and iron) can increase the amount and quality of essential oils in thyme (Jabbari et al., 2011); similarly nitrogen and sulfur have the same role in lemongrass (Zheljazkov et al., 2011). Nitrogen in plants is used to build amino acids and enzymes, which are utilized in the biosynthesis of various bioactive compounds (Nurzyńska-Wierdak, 2013). The degree of nitrogen fertilization is the main element for plant biomass accumulation and influences the quantity and proportion of different chemicals of secondary metabolites (Daneshian et al., 2009; Politycka & Golcz, 2004), also at *in vitro* plant cell or tissue cultures (Murthy et al., 2014a; Murthy et al., 2014b). Some researches indicated that nitrogen concentration and $NH4^+/NO3^-$ ratio affect the hypericins in *Hypericum* spp. (Cui et al., 2010; Murthy et al., 2014a). Also, hypericin content significantly varied depending on the developmental stage. Different harvest stages influence the hypericin content, whereas leaf hypericin content at earlier growth stages was higher than the fruit set stage (Sun et al., 2019). Also, *Hypericum triqutrifolium* Turra showed a higher accumulation of total hypericin at the vegetative stage than the flowering and fruiting stages of herb (Azeez et al., 2017). According to the results of research to investigate hypericins variation in *Hypericum triquetrifolium* during plant growth it was observed that hypericin content in leaves was higher at the reproductive stages (flowering), but in stems was higher at the vegetative stages (Ayan & Cirak, 2008). Another research about the ontogenetical variation of hypericin and hyperphorin in *H. perforatum* revealed that the total hypericin contents decreased as developmental stages progressed (Büter & Büter, 2002).

To produce plant secondary metabolites through *in vitro* culture, acquiring information about the biosynthesis of the secondary metabolites is essential to improve the production of chemical compounds such as hypericin and hyperphorin (Vattikuti & Ciddi, 2005). Moreover, regarding that the function of a major part of plant genome is unknown so far, the relationship between enzymes, signaling substances, and small molecules is still relatively unknown. To fully understand the regulation of metabolites biosynthesis and purposeful changes in gene expression more research is required (Deepak et al., 2007). The effect of various factors on the expression of the genes involved in the biosynthesis of the pharmaceutical compounds can be investigated by a quantitative real-time PCR (qPCR) approach (Pfaffl, 2001). The production of active ingredients in medicinal plants is controlled by genetic factors. However, limited information exists about the effect of fertilizer treatments on the relative expression profiling of the genes involved in the biosynthesis pathway of active ingredients of medicinal plants.

In the present study, the effects of nitrogen fertilizer at different times after irrigation were investigated on the expression of *hyp-1*, *HpPKS1* (polyketide synthase), and *HpPKS2* genes in leaf and root of *Hypericum* spp., before and after flowering using qPCR. This is the first report about the relative expression of some genes of *Hypericum* spp. under different levels of root nitrogen supply at different time intervals after irrigation.

2 METHODS AND MATERIALS

2.1 PLANT MATERIALS

The experiments were carried out with *H. perforatum* L. and *H. androsaemum* L:. *Hypericum* seeds were provided by the Iranian Biological Resource Center (IBRC), Tehran, Iran. The seeds were planted in pots containing farm soil (5 seeds / 4 l pot). The physicochemical properties of soil were: saturation percentage (32 %), electricity conductivity (1.2 103 ds m^{-1}), pH of paste (7.7), total neutralizing value (8 %), organic carbon (0.22 %), K available (212 ppm), P available (7.6 ppm) and soil texture (sandy clay loam).

2.2 PLANT CULTURE AND FERTILIZERS TREATMENTS

A factorial design based on completely randomized design (CRD) with three replications was used. Every pot with 5 plants was considered as a replication, namely, each treatment included three pots. The urea granular fertilizer was fertilized at 0, 0.5, and 1 mg l^{-1} in intervals at 24, 48, and 72 hours after irrigating. For RNA extraction, 0.3 g from pooled leaves or roots of three plants of each pot were used. Plant material sampling was accomplished before flowering (200 days after seed planting) and after flowering (300 days after seed planting) stage. Roots/leaves of 5 plants formed the plant material of an experimental unit (a replication of a treatment).

2.3 RNA EXTRACTION, PURIFICATION AND CDNA SYNTHESIS

Total RNA was extracted from leaf and root samples using the GeneAll RiboEx kit (BioFrontier, Korea) based on the manufacturer's protocol. Agarose gel electrophoresis and NanoDrop 1000 spectrophotometer (U.S.A) were used to determining RNA quality and integrity and checking the RNA quantity respectively. Samples of RNA were treated with RNase-free DNase Kit (Fermentas, Hanover, MD) to remove any contaminating genomic DNA, before cDNA synthesis. First-strand cDNA was synthesized from 5 µg of total RNA treated with DNase I using 200U MMLV reverse transcriptase (GREEN BIO GENE) according to the manufacturers' instructions.

2.4 PRIMER DESIGN AND REAL-TIME PCR

Primer design was carried out using online primer Quest software, the primer sequences for *hyp-1*, *HpPKS1,* and *HpPKS2* genes are listed in Table 1. According to scientific reports, the 18s rRNA gene was used as the reference gene for data normalization. In the qPCR method, increasing or decreasing of expression quantity of genes is important, which calculated by the delta-delta method (Pfaffl, 2001). The qPCR was performed in a total volume of 20 µl containing 2 µl of cDNA, 10 µl SYBR Green master mix, and 4 pmol of each primer. The amplification reactions were carried out in a Step-One qPCR System (Applied Biosystem, ABI, USA) under the following conditions: 5 min at 95 °C, 35 cycles of "95 °C for 30 s, annealing temperature (Table. 1) for 30 s and 72 °C for 40 s", and 72°C for 10 s. All amplification reactions were repeated three times under identical conditions and included a negative control. To confirm that the PCR products were produced from cDNA and not genomic DNA, appropriate control reactions (without the existence of reverse transcriptase) were carried out. This experiment was carried out with two biological and three technical repeats.

2.5 STATISTICAL ANALYSIS

The data normality was confirmed through the Kolmogorov-Smirnov test, then the relative expression levels of the genes were calculated by ΔΔCT and the Relative Expression Software Tool (REST) software (Pfaffl, 2001) was used to data analysis. One-way ANOVA and mean comparisons (Duncan's test) was accomplished using SAS software.

3 RESULTS

3.1 RELATIVE EXPRESSION OF *HYP-1* GENE

Mean comparisons of *hyp-1* gene expression revealed significant differences among treatments. The mean expression of *hyp-1* gene was lower in control treatments (1.3-fold) compared to the nitrogen supporting treatments. Three the highest relative expressions of *hyp-1* belonged to the root of *H. perforatum*: AF1-72 (13.6-fold), BF1-72 (11.3-fold), AF0.5-72 (10.4-fold). In

Table 1: Primer sequences for *hyp-1, HpPKS1,* and *HpPKS2* genes used in the quantitative real-time PCR for gene expression analysis of *Hypericum* spp.

Gene		Primer sequence (5'-3')	Annealing temperature	Fragment size
hyp-1	Forward	TCCTGAGAGGAAGTACAGTGTC	58	125
	Reverse	AGACGGCCCAGATTCATCA		
HpPKS1	Forward	GACAGAGGTGTGGAAGAAG	63	112
	Reverse	GTACATCAACTGCGCCAT		
HpPKS2	Forward	CCTTCCCTTCCGTGTGTATATG	63	184
	Reverse	CAGAGGAGAAACTGGAGGAAG		
18srRNA	Forward	TGTCTGCGATAATGGAACTG	58	108
	Reverse	ATTCATCATACTCCGCCTTAGC		

all cases, increasing the interval between urea fertilization and irrigation caused an increasing trend in gene expression level ($p < 0.01$). Furthermore, in all cases, by increasing the amount of urea fertilizer, the relative gene expression was increased ($p < 0.01$). In overall, it can be concluded that *hyp-1* relative expression in root tissue was greater than in leaf tissues ($p < 0.01$) (Figure 1).

3.1.1 Leaf

Among the leaf samples, the highest relative expression of the *hyp-1* gene belonged to the treatment of [Hp]AF1-72 (10-fold), the second to fourth highest *hyp-1* expression was observed in [Hp]AF1-48 (7.6-fold), [Hp]AF0.5-72 (7.5-fold) and [Ha]AF1-72 (7.4-fold) treatments respectively, and the next-highest gene expression was assigned to the treatment [Ha]BF1-72 (4.5-fold). In general, the mean expression level of *hyp-1* in *H. androsaemum* leaves (3.3-fold) was lower than in *H. perforatum* leaves (5.1-fold; $p < 0.01$). The lowest average expression of *hyp-1* was observed at before flowering sampled leaves of *H. androsaemum* (2.3-fold). The mean expression level in after flowering samples (5.1-fold) was higher than in before flowering (3.3-fold), so the effect of time on *hyp-1* expression in *Hypericum* leaves was significant ($p < 0.01$) (Figure 1).

3.1.2 Root

Among the root samples, the top three *hyp-1* relative expressions were [Hp]AF1-72 (13.6-fold), [Hp]BF1-72 (11.3-fold), and [Hp]AF0.5-72 (10.4-fold). Fourth to sixth the highest *hyp-1* expression belonged to [Ha]AF1-72 (9.7-fold) and [Hp]AF1-48 (9.5-fold), [Hp]BF1-48 (8.9-fold) and [Ha]BF1-72 (8.9-fold), respectively. In general, *hyp-1* mean expression in *H. androsaemum* roots (5.5-fold) was lower than in *H. perforatum* (7.1-fold), and the lowest mean expression level was observed in before flowering samples of *H. androsaemum* root tissues (5.1-fold). The relative expression of *hyp-1* in after flowering samples (6.8-fold) was higher than in before flowering samples (5.8-fold), so the effect of time on *hyp-1* expression in both *Hypericum* species roots is significant ($p < 0.01$) (Figure 1).

3.2 RELATIVE EXPRESSION OF *HPPKS1* GENE

Duncan's test for *HpPKS1* expression level revealed significant differences among means of treatments. The mean expression of *HpPKS1* gene in control treatments (0.7-fold) was lower than in the nitrogen fertilized treatments. The two highest relative expressions of *HpPKS1* belonged to leaves of *H. perforatum*: AF1-72 (9.1-fold) and BF1-72 (8-fold). The third to the seventh mean expressions of *HpPKS1* belonged to the following treatments of leaf samples, respectively: [Hp]AF1-48 (6.2-fold), [Hp]BF1-48 (6-fold), [Hp]AF0.5-72 (5.6-fold), [Ha]AF1-72 (5.2-fold) and [Hp]BF0.5-72 (5-fold). In all cases, with increasing the interval between urea fertilization and irrigation, the relative expression of *HpPKS1* had an increasing trend ($p < 0.01$). Furthermore, in all cases, by increasing the amount of urea fertilizer, *HpPKS1* expression was increased ($p < 0.01$). In overall, it can be concluded that *HpPKS1* mean expression in leaf was very greater than in root ($p < 0.01$) (Figure 2).

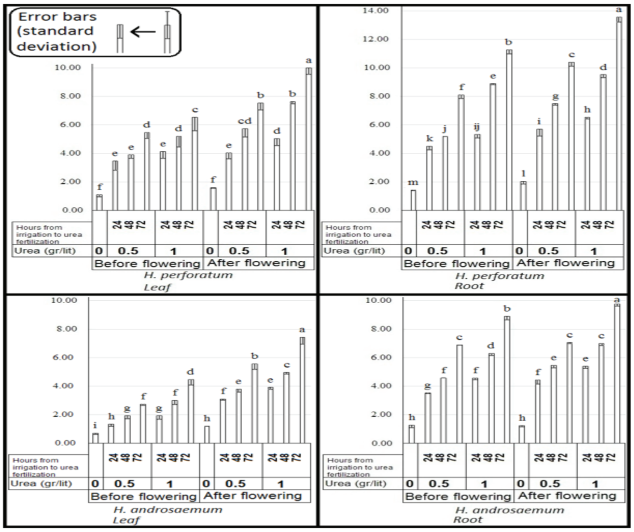


Figure 1: *hyp-1* expression level in different parts of *Hypericum* leaf and root (r = 3; $p < 0.05$)

3.2.1 Leaf

Among the leaf samples, the top four *HpPKS1* expression were belonged to *H. perforatum*: AF1-72 (9.1-fold), BF1-72 (8-fold), AF1-48 (6.2-fold) and BF1-48 (6-fold). In general, *HpPKS1* expression in *H. androsaemum* leaves (2.9-fold) was lower than in *H. perforatum* (4.6-fold; $p < 0.01$).,The lowest mean expression of *HpPKS1* was observed at before flowering samples of *H. androsaemum* leaf tissues (2.5-fold). *HpPKS1* mean expression in after flowering samples (4-fold) was higher than in before flowering (3.4-fold; $p < 0.01$), thus the developmental stage significantly affected *HpPKS1* expression level in both *Hypericum* species leaves (Figure 2).

3.2.2 Root

Among the root samples, the two highest *HpPKS1* expressions have belonged to [Hp]AF1-72 (3.3-fold) and [Ha]AF1-72 (2.6-fold). *HpPKS1* mean expression level was approximately equal in two *Hypericum* species root tissues (1.6-fold). The relative gene expression in after flowering samples (1.8-fold) was higher than in before flowering samples (1.5-fold; $p < 0.01$) (Figure 2).

3.3 RELATIVE EXPRESSION OF *HPPKS2* GENE

Based on Duncan's mean comparisons for *HpPKS2* expression level discovered significant differences among various treatments. The mean expression of *HpPKS2* gene in control treatments (0.8-fold) was lesser than in the nitogen fertilized treatments. Among all treatments, the top seven *HpPKS2* relative expressions belonged to the *H. perforatum* leaves: AF1-72 (8.4-fold), AF0.5-72 (7.8-fold), BF1-72 (7.6-fold), AF1-48 (6.4-fold), BF1-48 (6.3-fold), AF1-24 (5.3-fold), and BF0.5-72 (5.3-fold). In all cases, with increasing the interval between urea fertilization and irrigation, *HpPKS2* expression had an increasing trend ($p < 0.01$). Similarly, in all cases except two treatment, by increasing the amount of urea fertilizer, *HpPKS2* expression was increased ($p < 0.01$).

In overall, it can be concluded that the relative expression of the *HpPKS2* in leaves(3.9-fold) was very greater than in root (1.6-fold; $p < 0.01$) (Figure 3).

3.3.1 Leaf

Among the leaf samples, the top three *HpPKS2* relative expression treatments were [Hp]AF1-72 (8.4-fold), [Hp]AF0.5-72 (7.8-fold), and [Hp]BF1-72 (7.6-fold). In general, *HpPKS2* expression in *H. androsaemum* leaves (2.9-fold) was lower than in *H. perforatum* leaves (4.8-fold; $p < 0.01$). The lowest *HpPKS2* mean expression was observed at the before flowering samples of *H. androsaemum* (2.5-fold). *HpPKS2* mean expression in after flowering samples (4.3-fold) was higher than in before flowering (3.4-fold; $p < 0.01$), therefore the effect of developmental stage on *HpPKS2* expression in both *Hypericum* species leaves was significant (Figure 3).

3.3.2 Root

Among the root samples, the highest *HpPKS2* expression belonged to the treatment of [Hp]AF1-72 (3.1-

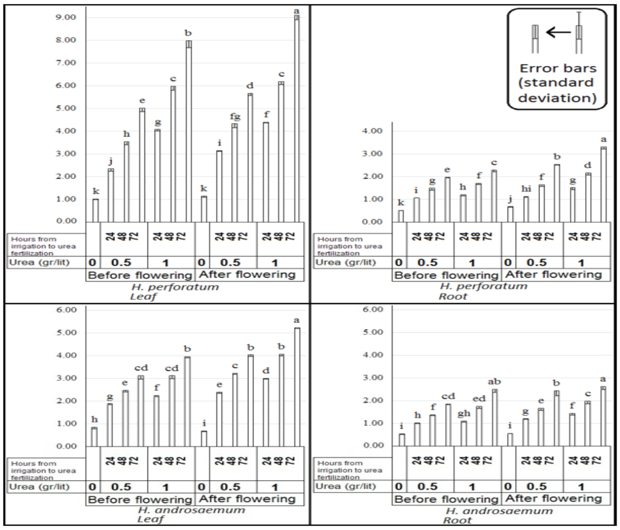


Figure 2: *HpPKS1* expression level in different parts of *Hypericum* leaf and root (r = 3; $p < 0.05$)

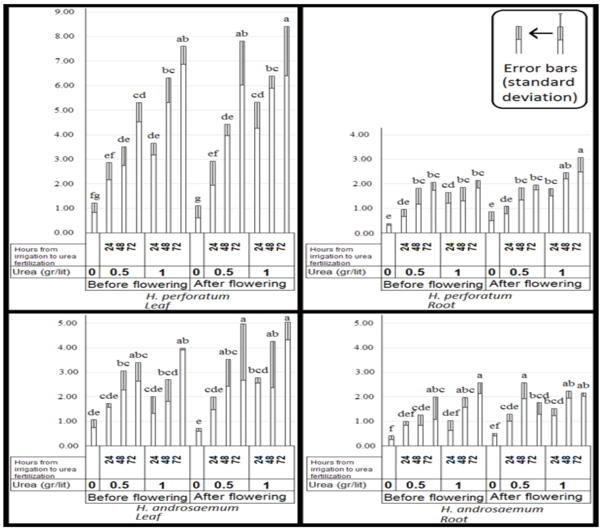


Figure 3: *HpPKS2* expression level in different parts of *Hypericum* leaf and root (r = 3; $p < 0.05$)

fold), then the treatment [Ha]BF1-72 (2.6-fold). The relative expression of the *HpPKS2* gene was approximately equal in two *Hypericum* species root tissues (1.6-fold). *HpPKS2* expression in after flowering samples (1.8-fold) was slightly higher than in before flowering (1.5-fold; $p < 0.01$) (Figure 3).

4 DISCUSSION

Production of secondary metabolites is controlled by the level of genes expression and is significantly influenced by the degree of cell differentiation, environmental conditions and the elements that are present in the plant (Vom Endt et al., 2002; Afrin et al., 2015). Lazzara et al. (2017) with the investigation of the effect of arbuscular mycorrhizal fungi (AFM) and phosphorus availability on the content of some secondary metabolites in *Hypericum perforatum*, reported that flower hyperforin concentration in mycorrhizal treatment was 17.5 % lower than non-mycorrhizal treatment. In contrast, with the use of AFM under low phosphorus availability, hypericin and pseudochypericin concentrations increased by 166.8 % and 279.2 %, respectively, while no effect of the use of AFM was found under high phosphorus availability. Based on the *H. perforatum* field study to determine the influence of N and P fertilizers on the flavonoids content, it was concluded that 125kg N ha^{-1} and 50kg P_2O_5 ha^{-1} proved optimal for high dry herb yield and flavonoids content compared to unfertilized plants (Azizi & Dias, 2004). Also, in the present study, by increasing the amount of nitrogen fertilizer, the relative expression of the genes involved in the hypericin biosynthesis pathway was increased, which was statistically significant in most cases.

The ultimate steps of hypericin biosynthesis have been proposed to be accompanied by Hyp-1, a phenolic coupling protein, a member of genes encoding plant pathogenesis-related class 10 protein family (PR-10) (Bais et al., 2003; Radauer et al., 2008; Michalska et al., 2010). Like other PR-10 family proteins, *hyp-1* looks to be developmentally regulated (Pinto et al., 2005, Liu & Ekramoddoullah, 2006). Also in other research, leaf and intact roots showed the highest expression of *hyp-1*, while stems and old parts of roots presented significantly lower expression levels, hence, there is variable existence of Hyp-1 protein in leaves at different phases of leaf development. (Karppinen, 2010). In the present study, the relative expression of all three genes studied in most cases in the after-flowering stage was higher than in the before-flowering stage.

Diverse expression levels of *hyp-1* in different tissues and organs of *Hypericum* have been reported. Košuth et al. (2007) reported absence of differences in *hyp-1* expression between leaf margins and leaf inner parts. According to their findings from *ex vitro* plants, fairly high expression of *hyp-1* gene was measured in roots. In the present study, the relative expression difference between root and leaf in the case of the *hyp-1* gene was smallerthan that of *HpPKS1* and *HpPKS2* genes. According to some studies, the *HpPKS2* expression and the hypericins content were the highest in the parts containing the dark glands, such as the flower buds, and were low in the parts free of dark glands, such as stem and root. In the case of the *hyp-1* gene, the relative expression in the root tissue was higher than the corresponding treatments in the leaf tissue ($p < 0.01$), but the relative expression of *HpPKS1* and *HpPKS2* genes, in the root tissue, was lower than the corresponding treatments in the leaf tissue. It can be because of that the *hyp-1* gene, apart from contributing to the hypericins biosynthesis pathway, has other functions, and probably these biological roles are related to root tissue in *Hypericum* species. Because of the high expression of *HpPKS2* in the reproductive organs and as well in leaf borders, this gene may play a role in the agglomeration of defensive chemicals, such as hypericins (Agostinis et al., 2002; Onelli et al., 2002; Sirvent & Gibson, 2002; Sirvent et al., 2003).

Also, a research was conducted to find suitable sites for the synthesis of hypericin based on the level of *hyp-1* gene expression in the early stages of seedling development, and comparing the level of *hyp-1* gene expression in different parts of the plant regarding the presence or absence of dark glands. It has been found that *hyp-1* copies are found throughout the leaf tissue, but there is no significant difference in the level of gene expression in the margins and the inner parts of the leaf (Karppinen, 2010).

These results showed that *hyp-1*, *HpPKS1,* and *HpPKS2* expression in *Hypericum* significantly affected by the plant tissues, plant genus, amount and time of urea granular fertilization, and developmental phase of sampling.

5 CONCLUSIONS

In general, the results showed that using the urea fertilizer significantly affect the relative expression of studied genes, with increasing the urea fertilizer, relative expression of the studied genes increased. Gene expression values after flowering were higher than before flowering. Enhancing the time interval between irrigation and urea fertilizer application, gene expression increases. The gene *hyp-1* expression in root was higher than the leaf, but the relative expression of *HpPKS1* and *HpPKS2* genes in leaf was higher than in roots.

6 REFERENCES

Afrin, S., Huang, J. J., & Luo, Z. Y. (2015). JA-mediated transcriptional regulation of secondary metabolism in medicinal plants. *Science Bulletin*, *60*(12), 1062-1072. https://doi.org/10.1007/s11434-015-0813-0

Agostinis, P., Vantieghem, A., Merlevede, W., & de Witte, P. A. (2002). Hypericin in cancer treatment: more light on the way. *The international journal of biochemistry & cell biology*, *34*(3), 221-241. http://dx.doi.org/10.1016/S1357-2725(01)00126-1

Ayan, A. K., & Cirak, C. (2008). Variation of hypericins in Hypericum triquetrifolium Turra growing in different locations of Turkey during plant growth. *Natural product research*, *22*(18), 1597-1604. https://doi.org/10.1080/14786410701838213

Azeez, H. A., Ameen, A. H., & Faqe, S. A. (2017). Variation in production of of *Hypericum triqutrifoliu* developmental stages. *Journal of zankoy sulaimani-A, 19*(2), 29-36. https://doi.org/10.17656/jzs.10608

Azizi, M., & Dias, A. (2004, September). Nitrogen and phosphorus fertilizers affect flavonoids contents of St. John's wort (*Hypericum perforatum* L.). In *Proc. 4th Int. Iran & Russia Conf. Shahrkurd, Iran* (pp. 8-10). Retrieved from: http://iirc.narod.ru/4conference/Fullpaper/20003.pdf

Bais, H. P., Vepachedu, R., Lawrence, C. B., Stermitz, F. R., & Vivanco, J. M. (2003). Molecular and biochemical characterization of an enzyme responsible for the formation of hypericin in St. John's wort (*Hypericum perforatum* L.). *Journal of Biological Chemistry*, *278*(34), 32413-32422. http://dx.doi.org/ 10.1074/jbc.M301681200

Birt, D. F., Widrlechner, M. P., Hammer, K. D., Hillwig, M. L., Wei, J., Kraus, G. A., ... & Wiemer, D. F. (2009). *Hypericum* in infection: Identification of anti-viral and anti-inflammatory constituents. *Pharmaceutical biology*, *47*(8), 774-782. http://dx.doi.org/10.1080/13880200902988645

Briskin, D. P., & Gawienowski, M. C. (2001). Differential effects of light and nitrogen on production of hypericins and leaf glands in *Hypericum perforatum*. *Plant Physiology and Biochemistry*, *39*(12), 1075-1081. http://dx.doi.org/10.1016/S0981-9428(01)01326-2

Bruni, R., & Sacchetti, G. (2009). Factors affecting polyphenol biosynthesis in wild and field grown St. John's Wort (Hypericum perforatum L. Hypericaceae/Guttiferae). *Molecules*, *14*(2), 682-725. http://dx.doi.org/10.3390/molecules14020682

Büter, K. B., & Büter, B. (2002). Ontogenetic variation regarding hypericin and hyperforin levels in four accessions of *Hypericum perforatum* L. *Journal of Herbs, Spices & Medicinal Plants*, *9*(2-3), 95-100. https://doi.org/10.1300/J044v09n02_14

Butterweck, V. (2003). Mechanism of action of St John's wort in depression. *CNS drugs*, *17*(8), 539-562. http://dx.doi.org/10.2165/00023210-200317080-00001

Cui, X. H., Murthy, H. N., Wu, C. H., & Paek, K. Y. (2010). Adventitious root suspension cultures of *Hypericum perforatum*: effect of nitrogen source on production of biomass and secondary metabolites. *In Vitro Cellular & Developmental Biology-Plant*, *46*(5), 437-444. http://doi.org/10.1007/s11627-010-9310-y

Daneshian, A., Gurbuz, B., Cosge, B., & Ipek, A. (2009). Chemical Components of Essential Oils from Basil (Ocimum basilicum L.) Grown at Different Nitrogen Levels. *International Journal of Natural & Engineering Sciences*, *3*(3). http://www.ijnes.org/index.php/ijnes/article/view/505/475

Deepak, S. A., Kottapalli, K. R., Rakwal, R., Oros, G., Rangappa, K. S., Iwahashi, H., ... & Agrawal, G. K. (2007). Real-time PCR: revolutionizing detection and expression analysis of genes. *Current genomics*, *8*(4), 234-251. http://doi.org/10.2174/138920207781386960

Duke, J. A. (2002). *Handbook of medicinal herbs*. CRC press. Inc. Boca Raton, Florida.

Duppong, L. M., Delate, K., Liebman, M., Horton, R., Romero, F., Kraus, G., ... & Chowdbury, P. K. (2004). The effect of natural mulches on crop performance, weed suppression and biochemical constituents of catnip and St. John's wort. *Crop science*, *44*(3), 861-869. http://doi.org/10.2135/cropsci2004.0861

Falk, H. (1999). From the photosensitizer hypericin to the photoreceptor stentorin—the chemistry of phenanthroperylene quinones. *Angewandte Chemie International Edition*, *38*(21), 3116-3136. https://doi.org/10.1002/(SICI)1521-3773(19991102)38:21<3116::AID-ANIE3116>3.0.CO;2-S

Jabbari, R., Dehaghi, M. A., Sanavi, A. M. M., & Agahi, K. (2011). Nitrogen and iron fertilization methods affecting essential oil and chemical composition of thyme (*Thymus vulgaris* L.) medical plant. *Advances in Environmental Biology*, 433-439. http://www.aensiweb.com/old/aeb/2011/433-438.pdf

Jendželovská, Z., Jendželovský, R., Kuchárová, B., & Fedoročko, P. (2016). Hypericin in the light and in the dark: two sides of the same coin. *Frontiers in plant science*, *7*, 560. http://dx.doi.org/10.3389/fpls.2016.00560

Karioti, A., & Bilia, A. R. (2010). Hypericins as potential leads for new therapeutics. *International journal of molecular sciences*, *11*(2), 562-594. http://dx.doi.org/10.3390/ijms11020562

Karppinen K (2010) Biosynthesis of hypericins and hyperforins in *Hypericum perforatum* (St. John's wort)-precursors and genes involved. Academic dissertation submitted to Faculty of Science, University of Oulu, Oulu. ISBN 978-951-42-6310-1. http://jultika.oulu.fi/Record/isbn978-951-42-6310-1

Khosh-Khui, M., Shekafandeh, A., & Azarakhsh, H. (1984). Micropropagation of myrtle. *Scientia horticulturae*, *22*(1-2), 139-146. https://doi.org/10.1016/0304-4238(84)90094-3

Košuth, J., Katkovčinová, Z., Olexová, P., & Čellárová, E. (2007). Expression of the hyp-1 gene in early stages of development of *Hypericum perforatum* L. *Plant cell reports*, *26*(2), 211-217. http://dx.doi.org/10.1007/s00299-006-0240-4

Kubin, A., Wierrani, F., Burner, U., Alth, G., & Grunberger, W. (2005). Hypericin-the facts about a controversial agent. *Current pharmaceutical design*, *11*(2), 233-253. http://dx.doi.org/10.2174/1381612053382287

Lazzara, S., Militello, M., Carrubba, A., Napoli, E., & Saia, S. (2017). Arbuscular mycorrhizal fungi altered the hypericin, pseudohypericin, and hyperforin content in flowers of *Hypericum perforatum* grown under contrasting P availability in a highly organic substrate. *Mycorrhiza*, *27*(4), 345-354. http://dx.doi.org/10.1007/s00572-016-0756-6

Lazzara, S., Napoli, E., & Carrubba, A. (2015). Hypericum spp.: a resource from wild Mediterranean flora for the treatment of mild depression. *Bioactive phytochemicals—perspectives for modern medicine*, *3*, 337-354.

Liu, J. J., & Ekramoddoullah, A. K. (2006). The family 10 of plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses. *Physiological and Molecular Plant Pathology*, *68*(1-3), 3-13. http://doi.org/10.1016/j.pmpp.2006.06.004

Michalska, K., Fernandes, H., Sikorski, M., & Jaskolski, M. (2010). Crystal structure of Hyp-1, a St. John's wort protein implicated in the biosynthesis of hypericin. *Journal of structural biology*, *169*(2), 161-171. http://doi.org/10.1016/j.jsb.2009.10.008

Miskovsky, P. (2002). Hypericin-a new antiviral and antitumor photosensitizer: mechanism of action and interaction with biological macromolecules. *Current drug targets*, *3*(1), 55-84. http://dx.doi.org/10.2174/1389450023348091

Murthy, H. N., Kim, Y. S., Park, S. Y., & Paek, K. Y. (2014a). Hypericins: biotechnological production from cell and organ cultures. *Applied microbiology and biotechnology*, *98*(22), 9187-9198. http://dx.doi.org/10.1007/s00253-014-6119-3

Murthy, H. N., Lee, E. J., & Paek, K. Y. (2014b). Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *118*(1), 1-16. http://dx.doi.org/10.1007/s11240-014-0467-7

Nürk, N. M., Madriñán, S., Carine, M. A., Chase, M. W., & Blattner, F. R. (2013). Molecular phylogenetics and morphological evolution of St. John's wort (*Hypericum*; Hypericaceae). *Molecular Phylogenetics and Evolution*, *66*(1), 1-16. http://doi.org/10.1016/j.ympev.2012.08.022

Nurzyńska-Wierdak, R. (2013). Does mineral fertilization modify essential oil content and chemical composition in medicinal plants? *Acta Sci Pol Hortorum Cultus, 12*(5), 3-16. http://www.acta.media.pl/pl/full/7/2013/000070201300012000050000300016.pdf

Onelli, E., Rivetta, A., Giorgi, A., Bignami, M., Cocucci, M., & Patrignani, G. (2002). Ultrastructural studies on the developing secretory nodules of *Hypericum perforatum*. *Flora-Morphology, Distribution, Functional Ecology of Plants*, *197*(2), 92-102. http://doi.org/10.1078/0367-2530-00019

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research*, *29*(9), e45-e45. http://doi.org/10.1093/nar/29.9.e45

Pinto, M. P., Ribeiro, A., Regalado, A. P., Rodrigues-Pousada, C., & Ricardo, C. P. P. (2005). Expression of *Lupinus albus* PR-10 proteins during root and leaf development. *Biologia plantarum*, *49*(2), 187-193. http://dx.doi.org/10.1007/s10535-005-7193-2

Politycka, B., & Golcz, A. (2004). Content of chloroplast pigments and anthocyanins in the leaves of *Ocimum basilicum* L. depending on nitrogen doses. *Folia horticulturae*, *16*(1), 23-29. http://www.ptno.ogr.ar.krakow.pl/Wydawn/FoliaHorticulturae/Spisy/FH2004/PDF16012004/fh1601p03.pdf

Radauer, C., Lackner, P., & Breiteneder, H. (2008). The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC evolutionary biology*, *8*(1), 286. http://doi.org/10.1186/1471-2148-8-286

Rahnavrd, A. (2017). Genetic and biochemical diversity of *Hypericum perforatum* L. Grown in the caspian climate of Iran. *Applied ecology and environmental research, 15*(1), 665–675. http://dx.doi.org/10.15666/aeer/1501_665675

Ruhfel, B. R., Stevens, P. F., & Davis, C. C. (2013). Combined morphological and molecular phylogeny of the clusioid clade (Malpighiales) and the placement of the ancient rosid macrofossil Paleoclusia. *International Journal of Plant Sciences*, *174*(6), 910-936. http://dx.doi.org/10.1086/670668

Russo, E., Scicchitano, F., Whalley, B. J., Mazzitello, C., Ciriaco, M., Esposito, S., ... & Mammì, M. (2014). *Hypericum perforatum*: pharmacokinetic, mechanism of action, tolerability, and clinical drug–drug interactions. *Phytotherapy research*, *28*(5), 643-655. http://doi.org/10.1002/ptr.5050

Samuelsson, G., & Bohlin, L. (2017). *Drugs of natural origin: a treatise of pharmacognosy* (No. Ed. 7). CRC Press Inc.

Silva, B. A., Ferreres, F., Malva, J. O., & Dias, A. C. (2005). Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. *Food chemistry*, *90*(1-2), 157-167. http://doi.org/10.1016/j.foodchem.2004.03.049

Sirvent, T., & Gibson, D. (2002). Induction of hypericins and hyperforin in *Hypericum perforatum* L. in response to biotic and chemical elicitors. *Physiological and Molecular Plant Pathology*, *60*(6), 311-320. http://doi.org/10.1006/pmpp.2002.0410

Sirvent, T. M., Krasnoff, S. B., & Gibson, D. M. (2003). Induction of hypericins and hyperforins in *Hypericum perforatum* in response to damage by herbivores. *Journal of chemical ecology*, *29*(12), 2667-2681. http://dx.doi.org/10.1023/B:JOEC.0000008011.77213.64

Sun, P., Kang, T., Xing, H., Zhang, Z., Yang, D., Zhang, J., ... & Li, M. (2019). Phytochemical Changes in Aerial Parts of *Hypericum perforatum* at Different Harvest Stages. *Records of Natural Products*, *13*(1). http://dx.doi.org/10.25135/rnp.77.18.04.267

Vattikuti, U. M., & Ciddi, V. (2005). An overview on *Hypericum perforatum* L. *Natural Product Radiance,* 4(5), 368-381. https://pdfs.semanticscholar.org/2a92/04ef4e20712d0613d3fdfee5c5bfec002362.pdf

Vom Endt, D., Kijne, J. W., & Memelink, J. (2002). Transcription factors controlling plant secondary metabolism: what regulates the regulators?. *Phytochemistry*, *61*(2), 107-114. http://doi.org/10.1016/S0031-9422(02)00185-1

Zhang, X., Jin, B., Zheng, W., Zhang, N., Liu, X., Bing, T., ... & Shangguan, D. (2016). Interaction of hypericin with guanine-rich DNA: Preferential binding to parallel G-Quadruplexes. *Dyes and Pigments*, *132*, 405-411. http://doi.org/10.1016/j.dyepig.2016.05.009

Zhang, Y., Shang, K., Wu, X., Song, S., Li, Z., Pei, Z., & Pei, Y. (2018). Highly efficient green synthesis and photodynamic therapeutic study of hypericin and its derivatives. *RSC advances*, *8*(39), 21786-21792. http://dx.doi.org/10.1039/c8ra03732a

Zheljazkov, V. D., Cantrell, C. L., Astatkie, T., & Cannon, J. B. (2011). Lemongrass productivity, oil content, and composition as a function of nitrogen, sulfur, and harvest time. *Agronomy Journal*, *103*(3), 805-812. http://doi.org/10.2134/agronj2010.0446

Zobayed, S. M. A., Afreen, F., Goto, E., & Kozai, T. (2006). Plant–environment interactions: accumulation of hypericin in dark glands of *Hypericum perforatum*. *Annals of Botany*, *98*(4), 793-804. http://doi.org/10.1093/aob/mcl169

doi:10.14720/aas.2020.116.1.1652

Review article / pregledni znanstveni članek

The role of far-red light (FR) in photomorphogenesis and its use in greenhouse plant production

Bojka KUMP [1, 2]

Received May 08, 2020; accepted May 30, 2020.
Delo je prispelo 08. maja 2020, sprejeto 30. maja 2020.

The role of far-red light (FR) in photomorphogenesis and its use in greenhouse plant production

Abstract: Light energy is one of the most important factors regulating the growth and development of plants. In greenhouses and other controlled- environments in which the natural radiation intensities are often low, plant production relies on supplementary lighting to optimize the photosynthesis, increase production levels, and enable year-round production. For a long time, the research related to artificial lighting sources focused on the optimization of the efficiency of use for photosynthesis. The quality of light in plant production has been widely addressed only recently with the development of advanced LED technology that is energy efficient and enables the control of the spectral composition of light. Red and far-red light are sensed by the phytochromes that trigger several morphological and developmental processes that impact productivity and yield quality. Thus, to efficiently exploit all the advantages of LEDs and to develop LED arrays for specific plant applications, it is essential to understand thoroughly how light quality influences plant growth and development. This paper presents an overview of the recent developments in light quality manipulation, focusing on far-red light and the R: FR ratio, to improve yield and quality of products and to manage plant architecture and flowering in vegetable and ornamental horticulture.

Key words: far-red light; greenhouse; LEDs, light quality; photomorphogenesis; R: FR ratio

Vloga dolgovalovne rdeče svetlobe v fotomorfogenezi in njena uporaba pri vzgoji rastlin v rastlinjakih

Izvleček: Svetlobna energija je eden najpomembnejših dejavnikov, ki uravnava rast in razvoj rastlin. V rastlinjakih in ostalih zavarovanih prostorih, kjer je jakost naravnega sevanja velikokrat majhna, je vzgoja rastlin odvisna od dosvetljevanja, ki omogoča optimizacijo fotosinteze, povečanje pridelka in celoletno vzgojo rastlin. Dolgo časa so se raziskave povezane z umetnimi viri osvetljevanja osredotočale predvsem na izboljšanje učinkovitosti za fotosintezo. Vloga kvalitete svetlobe pri rastlinski pridelavi je postala zanimiva šele pred kratkim, z razvojem energetsko učinkovite, napredne LED tehnologije, katere glavna prednost je, da omogoča nadzor nad spektralno sestavo svetlobe. Rdeča in dolgovalovna rdeča svetloba, ki jo rastline zaznavajo s fotoreceptorji fitokromi, sprožita številne morfološke in razvojne procese, ki vplivajo na količino in kvaliteto pridelka. Za dober izkoristek vseh prednosti LED tehnologije in razvoj "LED matrik" za specifično rastlinsko uporabo, je potrebno dobro razumevanje vpliva spektralne sestave svetlobe na rast in razvoj rastlin. V prispevku je predstavljen pregled napredka na področju upravljanja s svetlobo, s povdarkom na dolgovalovni rdeči svetlobi in razmerju med rdečo in dolgovalovno rdečo svetlobo, za povečanje in izboljšanje kakovosti pridelka, ter uravnavanje zgradbe rastlin in cvetenja v pridelavi vrtnin in okrasnih rastlin.

Ključne besede: dolgovalovna rdeča svetloba; rastlinjak; LED luči; kvaliteta svetlobe; fotomorfogeneza; R : FR razmerje

---

[1] University of Ljubljana, Biotechnical Faculty, Agronomy Department, Ljubljana, Slovenia
[2] Corresponding author, e-mail: bojka.kump@bf.uni-lj.si

1 INTRODUCTION

Many food and ornamental horticultural crops are produced year-round in greenhouses. Recently, plant factories and vertical farms that can ensure a supply of locally produced, fresh food are also being introduced (Bantis et al., 2018). The availability of natural sunlight in these controlled-environments is often limited and could prevent or suppress plant production during large parts of the year. In such circumstances, the production of many crops rely on artificial lighting

In greenhouses, traditional artificial lighting sources, such as fluorescent, high-pressure sodium, metal halide and incandescent lamps, have long been used as a solo source or supplemental lighting to enhance photosynthesis and/or to regulate plant development (Bilodeau et al., 2019; Bantis et al., 2018). The spectral qualities of artificial light sources differ with each other and from natural light. Metal halide and especially high-pressure sodium lamps (HPS) have been usually used to provide high-intensity lighting for plant growth applications.

Recently developed lighting source in the form of light-emitting diodes (LEDs) offers many advantages over conventional artificial lighting sources (Chang et al., 2012; Olle & Viršile, 2013; Morrow, 2008; Yeh & Chung, 2009; Singh et al., 2015; Cho et al., 2017; Viršilė et al., 2017). Advantages include high-energy efficiency, long operating lifetime, narrow spectral emissions, adjustable size, etc. (Singh et al., 2015; Nelson & Bugbee, 2014). LEDs also produce significantly lower leaf temperatures and are thus suitable for low-intensity lighting within the plant canopies (Massa et al., 2005). One of the most valuable properties of LEDs is the ability to control the spectral composition of supplemental lighting, which enables investigating the effects of individual wavelengths or different spectral combinations of wavelengths on plant growth and development. The first commercially available LED fixtures were mainly designed to support photosynthesis and contained mostly red and blue diodes (10 %-25 % blue diodes). To date, many experiments have focused on designing an optimal lighting system, mostly modifying the proportion of different wavelengths within photosynthetically active radiation (PAR) region to support plant growth (Massa et al., 2008) and, more recently, also to precisely regulate plant development. The spectral quality of light affects a great number of plant developmental processes, including germination, seedling establishment, development of rosette, branching and apical dominance, the definition of adult plant architecture, the regulation of flowering, etc. (Franklin & Quail, 2010; Sánchez-Lamas et al., 2016).

The objective of this review is to present a short overview of the principles of red (R) (≈ 625-700 nm) and far-red (FR) (> 700 nm) light perception by phytochromes and their impact on plant growth and development, which play an essential role in greenhouse plant production. We summarise the research work referring to the use of far-red light or manipulating the R : FR ratio, to improve yield and quality of products and to manage plant architecture and flowering in vegetable and ornamental horticulture.

2 RADIATION AND PLANTS

About 40-50 % of the solar energy received by ecosphere falls in the spectral region of 380-780 nm, which we perceive as visible light. The visible light range includes violet (≈ 400-450 nm), blue (≈ 450-520 nm), green (≈ 520-560 nm), yellow (≈ 560-600 nm), orange (≈ 600-625), red (≈ 625-700 nm) and far-red (> 700 nm). On the short-wavelength end of this region is ultraviolet radiation (UV-A, 315-380 nm; UV-B, 280-315 nm; UV-C from artificial sources of radiation, 100-280 nm) and, at the other end, there is infrared radiation (780-3000 nm). The radiation that hits the plant can be reflected at the surface, absorbed or transmitted; the degree of these three events depends on the wavelength of the radiation (Larcher, 1991) and plant organs structure. For a plant, radiation is the energy source and a stimulus regulating development, but it can also cause photo-inhibitory and photo-destructive effects.

In photo-energetic processes, the absorbed energy is used for the photosynthesis or other metabolic reactions or chemical transformations. The action spectrum of plant leaves was described as the span of wavelengths from approximately 400-700 nm, over which plants absorb and effectively use radiant light energy for photosynthesis (McCree, 1972). This photosynthetically active radiation, now commonly known as PAR, is the most important part of the light spectrum for plants; it falls within the visible light range. It relates the intensity and rate of radiant light energy per surface area emitted by a light source from within the action spectrum of plants (Bilodeau et al., 2019). The amount of light that reaches the plant within the PAR region, or the number of photosynthetically active photons that fall on a given surface each second, is measured as a photosynthetic photon flux density (PPFD) and is expressed in μmol m^{-2} s^{-1}. The photoreceptors that play an important role in photosynthesis are chlorophylls with absorption maxima in the red and blue, along with antenna pigments that include different carotenoids, such as β-carotene, zeaxanthin, lycopene, and lutein (Singh et al., 2015).

Plants are also sensitive to other wavelengths, for example, far-red radiation of a wavelength of approximately 700-800 nm. It is known that the far-red part of spectrum strongly affects plant development. The regulation of plant growth and development by light signals is termed 'photomorphogenesis'. Recently, it was shown that far-red light could also increase photosynthetic efficiency (Zhen & van Iersel, 2017).

Plants respond to changes in light, including its intensity, spectral quality, direction and duration through different types of photoreceptors. Not considering chloroplast pigments, to date, five photosensory systems have been identified, (i) the phytochromes (phys) that absorb maximally in the red (600-700 nm) and far-red (700-750 nm) regions of the spectrum, (ii) the cryptochromes (crys), phototropins (phots) and members of the Zeitlupe family (ztl, fkf1 and lkp2) that absorb the blue light (390-500 nm) and (iii) UV resistance locus 8 (UVR8) that monitors the ultraviolet B wavelengths (280-315 nm) (Bantis et al., 2018).

3 PHYTOCHROMES

Phytochromes are light-sensing molecules present in bacteria, cyanobacteria, fungi, algae, and land plants. The first indications of their role in plant development came from studies in the 1930s, although its chemical isolation and identification happened much later (Taiz & Zeiger, 2014).

Native phytochrome is a soluble protein with a molecular mass of about 250 kDa. It occurs as a dimer made up of two equivalent subunits. Each subunit consists of a light-absorbing pigment molecule called the 'chromophore' that is synthesized in chloroplasts and a polypeptide chain called 'apoprotein'**,** synthesized in the cytosol. Together they form the holoprotein, which can absorb light (Taiz & Zeiger, 2014; Lagarias & Rapoport, 1980).

Phytochrome exists in two interconvertible forms that differ in their absorption spectra. The inactive Pr form is synthesized in the dark and after the absorption of red light (660 nm) changes chemically into the active Pfr form. Pfr is inactivated by the conversion back to Pr after the absorption of far-red light (730 nm) or through thermal relaxation, which depends on temperature. (Taiz & Zeiger, 2014; Legris et al., 2019). Phytochromes act as dimers and subunits are connected in three possible combinations Pr-Pr, Pfr-Pr, and Pfr-Pfr.

The photo-reversibility is the most distinctive property of phytochrome and results in a dynamic photoequilibrium of Pr and Pfr that depends on light quantity, spectral composition and temperature (Franklin and Quail, 2010; Jung, 2016). The absorption of light by Pr induce an extensive variety of different responses that can be grouped into two types: (i) rapid biochemical events and (ii) slower morphological changes, including movements and growth (Taiz & Zeiger, 2014).

Phytochrome is encoded by a small multigene family named *PHY*, the apoprotein without chromophore is designated 'PHY', and the holoprotein is designated 'phy' (Taiz & Zeiger, 2014). Phytochromes in seed plants diverged into three major forms, phyA, phyB, and phyC, very early in the history of seed plants. In some dicotyledonous species, two additional forms, phyE and phyD, were found and are presumably derived by gene duplication. Each phytochrome has different roles, and their relative contributions vary depending on the environmental conditions and developmental stage of the plant (Legris et al., 2019). PhyA and phyB are present in all sampled angiosperms and are the principal mediators of red/far-red–induced responses.

Research of the expression patterns of the *PHY* gene family of products has shown that there are two different classes of phytochromes with distinct properties: light labile Type I and light stable Type II (Tokuhisa et al., 1985; Furuya, 1993). Type I class, represented by phyA, induces very low fluence responses (VLFRs) and far-red high-irradiance responses (FR-HIR). The examples of conditions where phyA is activated are deep shade or few centimetres under the soil surface. Type II phytochromes (phyB-phyE) evoke signals called 'low fluence responses' (LFRs), and these are activated in more open environments (Legris et al., 2019).

Following light perception and conversion to the Pfr form, phytochromes translocate to the nucleus where they selectively interact with several classes of transcription factors and trigger downstream signalling, participating in the regulation of transcription posttranscriptional regulation, and translation (Legris et al., 2019).

In addition to light, phytochromes also contribute to temperature responses with phyB having a major role (Jung et al., 2016; Legris et al., 2019). The molecular mechanisms of photo- and thermo-morphogenesis have mostly been analysed separately, but it is now well recognised that light and temperature signalling are connected (Lorenzo et al., 2016; Patel et al., 2013).

Phytochromes can act synergistically with other photoreceptors. The interaction of phytochrome and cryptochrome signalling and interaction between phytochrome and phototropin photoreceptors have been reported in *Arabidopsis* (Ahmad et al., 1998; Mas et al., 2000; Parks et al., 1996).

3.1 PHYTOCHROMES AND R : FR RATIO IN PHOTOMORPHOGENESIS

Pr and Pfr forms of phytochromes have overlapping absorption spectra and can monitor the R : FR ratio of light. The R : FR ratio is usually used to quantify spectral photon flux distribution in the R and FR wavelengths. The equilibrium between the Pr and Pfr forms dynamically changes with the composition of the light spectrum within the 300-800 nm range and is strongly correlated with R to FR proportions of red and far-red light in incident radiation (Holmes & Smith, 1977). Sunlight at midday has approximately equal amounts of R and FR resulting in an R : FR ratio slightly above 1, but at the beginning and end of the photoperiod it is much lower (about 0.6). The R : FR ratio slightly varies with cloud covering and is little sensitive to seasonal variation (Turnbull & Yates, 1993). However, in environments with high plant density, the R : FR ratio is considerably reduced (0.13 under ivy canopy) (Taiz & Zeiger, 2014). In canopies, the R : FR ratio perceived by plant organs varies spatiotemporally in a range within which slight R : FR variation causes large variation in phytochrome photo-equilibrium (Demotes-Mainard et al., 2016).

Phytochromes are potent regulators of plant development and, through the perception of the R : FR ratio of light, affect a broad range of responses throughout the plant life cycle (Franklin & Quail, 2010; Sánchez-Lamas et al., 2016). The change in the R : FR ratio triggers a series of responses in plant vegetative architecture including higher stem length, bud outgrowth, leaf expansion, petiole extension, root development, leaf hyponasty and phototropism, accelerated flowering, etc., known collectively as 'shade avoidance syndrome' (SAS). Green tissues of plants with high chlorophyll content absorb mostly red (R) and blue (B) wavelengths, but let through the FR light, so with shading the R : FR ratio decreases. The greater proportion of far-red light converts more Pfr to Pr, and the ratio of active Pfr to total phytochrome (Pfr/P_{total}) decreases.

The shade-avoiding response is characteristic of 'sun plants' and represents a competitive advantage as it enables the plants to acquire more PAR. The sensitivity of plants to low R : FR ratio can vary during the day (Casal et al., 1997) and it also depends on the variety and species (Casal & Smith, 1989). The SAS response is weak or not present in 'shade plants', which normally grow in the shade (Taiz & Zeiger, 2014).

One of the SAS responses triggered by a low R : FR ratio is a faster transition to flowering. It is common in many plant species, including many agronomical significant ones (Taiz & Zeiger, 2014). The first step in flowering, the flower induction, is a result of the integration of various environmental and endogenous cues that work through different genetic pathways. The photoperiod pathway is one of them and refers to the regulation of flowering in response to day length and quality of light perceived (Srikanth & Schmid, 2011). On the basis of the photoperiod, plants can be classed as short-day (SDP) or long-day plants (LDP), although the critical factor in determining their activity is night length. Species that flower under any photoperiodic condition are referred to as 'day-neutral plants' (DNP).

4 APPLICATIONS OF FAR-RED LIGHT IN GREENHOUSE PLANT PRODUCTION

There are many areas in crop production, of both food crops and ornamental crops, for which the use of FR light has been shown to have especially promising value. In vegetable horticulture, the two main factors defining yield are biomass production and product quality, inclusive of nutritional value, visual appearance, resistance to 'environmental factors' and others. In growing ornamental plants, the main focuses are their visual appearance and flowering characteristics.

4.1 PHOTOSYNTHESIS AND BIOMASS PRODUCTION

Biomass production is usually directly correlated to photosynthesis, and it depends on the quantity of photosynthetically active radiation received by the leaves. The primary environmental factors that influence crop growth include photosynthetic light intensity and duration per day (which, when integrated formulates the photosynthetic daily light integral), light quality, temperature, and carbon dioxide concentration (Runkle, 2013).

Due to the poor absorption of far-red light ($\lambda >$ 700 nm) by leaves and the low quantum yield of photosynthesis, it has long been thought that it makes no or minimal contribution to photosynthesis (McCree, 1972). It is now known that the low quantum yield of photosynthesis under far-red light is caused by unbalanced excitation of the photosystems PSI and PSII, which operate in series to carry out photochemical reactions (Myers, 1971). If shorter wavelength light is supplemented with far-red light that preferentially excites PSI, the excitation balance between the two photosystems can be restored. This can synergistically increase photochemistry and photosynthesis (Zhen & Van Iersel, 2017).

Zhen and Van Iersel (2017) report that the addition of far-red light to red/blue and warm-white light immediately increased quantum yield of photosystem II (Φ_{PSII}) of lettuce by an average of 6,5 and 3 %, respectively. Besides the short-term effect, after 20 min of exposure to far-red light, an increase of Φ_{PSII} and a decrease of non-photochemical quenching (NPQ) were noted. The increase of Φ_{PSII} was associated with an increase in net photosynthesis. A 7.5 % increase in Φ_{PSII} upon adding far-red light (90 µmol m^{-2} s^{-1} within 700–770 nm) to a red/blue measuring light (200 µmol m^{-2} s^{-1}) resulted in an 18 % increase in net photosynthetic rate. In a later study, Zhen et al. (2019) quantified the efficiency of different wavelengths of far-red light exciting PSI by measuring the change in quantum yield of PSII of lettuce grown under red/blue light or under sunlight. The Φ_{PSII} of lettuce increased with increasing wavelengths of added light from 678 to 703 nm, indicating longer wavelengths within this region are increasingly used more efficiently by PSI than by PSII. Adding 721 nm light resulted in similar Φ_{PSII} as adding 703 nm light, but Φ_{PSII} tended to decrease as wavelength increased from 721 to 731 nm, likely due to decreasing absorptance and low photon energy. Adding 752 nm light did not affect Φ_{PSII}. The beneficial effects of FR light on photosynthesis at fluctuating light was also proved in *Arabidopsis* by Kono et al. (2020).

Photosynthesis is dependent on the intensity and quality of light that reaches chloroplasts. Yang et al. (2018) studied the interactions between light intensity and the R : FR ratio on the photosynthesis of soybean leaves under shade condition. From the results of quantitative proteomic analysis, they conclude that light intensity directly affects photosynthesis by regulating the expression of photosynthetic proteins and that a low R : FR ratio could improve the photosynthetic capacity by increasing light-harvesting capacity and stimulating the electron transport of PSI (Yang et al., 2018).

Whole-plant gas exchange measurements of 16 C_3 and two C_4 species under red/blue light and red/blue plus FR light indicated that adding far-red photons (10 – 30 % of PPFD) caused similar increases in the whole canopy photosynthetic rate as adding the same amount of red/blue photons (Zhen & Bugbee, 2019). To demonstrate that far-red photons are equally efficient at driving photosynthesis, the authors grew lettuce under spectra with or without 15 % of far-red photons at the same total photon flux, and the results at harvest showed 35 % higher dry biomass in treatments with far-red photons, which was almost entirely caused by enhanced radiation capture through leaf expansion.

There is, however, a variability among studies and, in some species, the specific net assimilation rate decreases in response to a low R : FR ratio or PHYB reduced expression. The different and sometimes contrasted responses between studies may reflect variability between species and genotypes, and/or variability in the growing conditions used (Demotes-Mainard et al., 2016 citations therein).

In addition to its direct effect on photosynthesis, the R : FR ratio can influence the process also indirectly, through its effect on morphological characters, for example, branching, leaf area and leaf orientation (Demotes-Mainard et al., 2016). A higher proportion of FR light, applied to some ornamental plant species has been shown to cause leaf expansion mediated by phytochromes, thus promoting canopy light interception and indirectly increasing plant growth during long-term cultivation (Park & Runkle, 2017).

Phytochrome B also regulates other traits involved in photosynthesis. It was shown to promote stomatal maturation in *Arabidopsis thaliana* (Kang et al., 2009), regulate the activity of some enzymes of the Calvin cycle (Bradbeer, 1971) and control the development of chloroplasts (Thiele et al., 1999).

4.2 FR AND YIELD QUALITY

The quality of the crop yield is an essential parameter for any grower. Its various aspects rely upon the light intensity and, to a larger extent, on light quality, which is the wavelengths perceived by the plant. With modifications of the light spectrum, the simultaneous increase in biomass and better quality of the product can be obtained. Most recent investigations are thus focused on both characteristics. A large number of studies in vegetable horticulture have been done using lettuce and tomato as a model species.

In a study in which lettuce was grown under cool white fluorescent lamp supplemented with R or FR LED, the plants exposed to FR light demonstrated enhanced fresh and dry mass, stem length, and leaf length and width, but the content of anthocyanin, carotenoid, and chlorophyll was reduced (Li & Kubota, 2009). Similarly, red leaf lettuce grown under R supplemented with FR light also exhibited lower anthocyanin concentration and antioxidant potential, whereas R light supplemented with blue (B) light caused the exact opposite results (Stutte et al., 2009).

Zhang et al. (2019a) performed three greenhouse experiments to determine the effects of supplemental lighting with different spectral quality on lettuce growth, colouration, and sensory attributes. Four hours of end-of-day (EOD) lighting of low intensity (2-5 µmol m^{-2} s^{-1}) had only minor effects on lettuce growth and quality attributes, but R + B LED supplemental lighting resulted in darker, redder, and less yellowish plants.

An essential role of FR light for improving the production of indoor cultivated lettuce has been demonstrated in an experiment done by Zou et al. (2019). Adding 50 µmol m^{-2} s^{-1} of FR light to 200 µmol m^{-2} s^{-1} of R + B, applied during the day (16 h) or as EOD (1 h), resulted in 39 % and 25 % increase in total biomass. Adding FR light resulted in 27-49 % larger plant leaf area with an open plant canopy, which facilitated a better light interception led to higher plant radiation use efficiency (RUE).

Studies of FR effects during the fruiting stage of crops are much less frequent than research in young plants, although the reports, especially in tomato, are now increasing. Apart from plant morphological alterations, changes in the R : FR ratio also lead to the redistribution of growth and dry matter within the plants, as indicated by an increased shoot/root ratio and soluble sugar levels under radiation with a low R : FR ratio. Kalaitzoglou et al. (2019) reported that additional FR light applied as continuous and EOD treatment increased total dry mass of tomato plants in the vegetative growth stage, as well as the fruit number per plant, fresh fruit weight per plant and average fruit fresh mass. Similarly, higher total plant dry mass and higher fruit yield in tomato grown under additional FR radiation were reported by Zhang et al. (2019b). In another study on tomatoes, the R and B + FR light treatment increased the production of soluble sugar, increased the dilution by soluble and storage compounds, and water reduction dilution, leading to a strong increase in glucose, fructose and sucrose concentration in the pericarp (Fanwoua et al., 2019). However, neither study provided sufficient insights on how additional FR increases fruit growth in the fruiting stage of the crop, which is a key step in understanding the FR induced yield improvement in fruit crops like tomatoes. Kim et al. (2019) also observed a significant decrease in water content of tomato fruits produced under FR light, together with a pronounced effect of FR light on biomass allocation between vegetative and reproductive structures. Long-term exposure of tomato plants to low R : FR was associated with reductions in leaf area, chlorophyll content, and vegetative shoot biomass fraction, increases in leaf thickness and fruit biomass, and acceleration of flowering and fruiting.

Recently, intra-canopy lighting, a technique that enables more even light distribution within the crop canopy, has been widely applied for high-wire fruit vegetable production in greenhouses. In a study of Zhang et al. (2019b), tomatoes were grown under intra-canopy R and B LEDs supplemented with overhead FR lights in three different duration regimens, (i) 12 hours during the day, (ii) 1.5 hours in the afternoon and (iii) 0.5 hour in the afternoon. The additional FR lighting led to increased stem elongation, higher leaf length: width ratio, increased leaf area, 9 %-16 % increase in total biomass and slightly lower soluble sugar content. The stimulation of growth and production were similar in all three treatments (Zhang et al., 2019b).

The effects of specific spectral compositions of light on various quality parameters are often species, or even cultivar specific, and should thus be determined for each species and application separately. The recent wide accessibility of LEDs and promising results on the effects of the FR light in some model species, widely opened the research of this topic in many other agronomical important species, like cucumber (Nishimura et al., 2012; Hao et al., 2012; Shibuya et al., 2016) spinach (Akutsu et al., 2017), kale (Meng et al., 2019), squash (Yang et al., 2012), basil (Meng & Runkle, 2019), etc.

Morphological changes induced by FR light, especially stem elongation, are often undesirable, but in some cases, the promotion of extension growth is essential. Producing seedlings with long hypocotyls is, for example, desired in vegetable grafting. Chia and Kubota (2010) used FR-rich incandescent lamps, without or with filters that reduced transmission of R light, to promote elongation of tomato seedlings. They obtained a 20 % increase in hypocotyl length under the R : FR ratio of 0.47 and 44 % at R : FR of 0.05. They concluded that only 2 to 4 mmol m^{-2}d^{-1} of FR light was needed to nearly saturate the promotion of elongation growth. There were no negative effects on dry mass, stem diameter, and plastochron index. The effectiveness of EOD FR lighting from a movable LED fixture on interspecific squash (*Cucurbita maxima* Duch. *x Cucurbita moschata* Duch. ex Poir) rootstock hypocotyl elongation was investigated by Yang et al. (2012).The extent of hypocotyl elongation under movable or stationary FR LED fixtures was statistically the same and was 55-69 % greater than non-treated control (Yang et al., 2012).

It is known that, in addition to a low R : FR ratio, shade avoidance responses are also regulated by the photosynthetic photon flux density (PPFD). In general, increasing PPFD decreases the magnitude of plant responses to the R : FR ratio. Growing sunflower (*Helianthus annuus* L.) seedlings at low R : FR ratio (0.85) at low and moderate PPFD (157 and 421 μmol m^{-2}s^{-1}) resulted in increased growth, but the growth response was attenuated under the higher PPFD (Kurepin et al., 2007). Yang et al. (2018) studied the effects of interactions between light intensity and R : FR ratio on the photosynthesis of soybean leaves under shade condition. The iTRAQ technique was utilized to analyse differentially expressed proteins involved in photosynthesis, and the results showed that low R : FR could improve the photosynthetic capacity of plants under shade conditions.

Light is also a critical environmental factor for the production of secondary metabolites in plants (Kopsell & Sams, 2013). It is known that phytochemicals play a significant role in delaying or inhibiting oxidative damage caused by free radicals. Spectral quality has a pronounced effect on accumulating secondary plant metabolites in controlled environment agriculture (Alrifai et al., 2019). The studies about the impact of FR light on the phytochemicals content are limited. As mentioned above, lettuce plants exposed to FR light demonstrated enhanced fresh and dry mass, stem length, and leaf length and width, but the content of anthocyanin, carotenoid, and chlorophyll was reduced (Li & Kubota, 2009); similarly, red leaf lettuce grown under R light supplemented with FR light also exhibited lower anthocyanin concentration and antioxidant potential (Stutte et al., 2009). In such cases, the benefits of adding FR light for increased biomass are tentative. In many studies, FR light was applied together with B and R light, and the overall effect of this light combination on secondary metabolites concentration was mostly stimulatory, while the contribution of FR alone was not evaluated (Alrifai et al., 2019). This effect could be explained by enhanced photosynthesis and, consequently, higher metabolism.

Light spectral quality can greatly affect the efficiency of the uptake of resources (water and nutrients) from the soil and affect the way of the plants to adjust the competition for soil resources (Sadras et al., 1989; Gundel et al., 2014). It was shown that the R: FR ratio affects different steps of plant nutrition that are critical for enhanced and sustainable crop production. It can affect root structure (De Simone et al., 2000; Gundel et al., 2014) or root growth (Klem et al., 2019). The establishment of a nitrogen-fixing symbiosis between legumes and rhizobia requires not only sufficient primary metabolites but also the sensing of the R : FR ratio (Suzuki et al., 2011). R : FR sensing also influences the arbuscular mycorrhizal (AM) symbiosis of a legume and non-legume through jasmonic acid and strigolactone signalling (Nagata et al., 2015). In hydroponically grown lettuce, far-red light stimulated uptake of N and K, Ca and Mg with the latter increasing by 27 %, 25 %, and 28 %, respectively, compared to plants grown under R and B light (Pinho et al., 2017).

Postharvest technology is another field in which the use of LED technology emerged as an efficient lighting source and its use, including FR light, can affect the nutritional and organoleptic parameters of vegetable plants and can be used to delay the senescence of green vegetables (Favre et al., 2018). The use of R light or light with high R : FR ratio has been shown to be the most effective in delaying senescence in broccoli (Favre et al., 2018). Similarly, tomato fruits exposed to LED light with a high R : FR ratio had increased firmness and higher titratable acidity compared to other darkness exposure or other LED lighting conditions (Nájera et al., 2018).

4.3 FR IN ORNAMENTAL HORTICULTURE (FLORICULTURE)

In growing ornamental plants, one of the essential points to consider is the shape of plants, of which stem elongation and branching are regarded as very important. Another vital component is flowering, including flowering time and duration, and the number of floral buds. All of these contribute to the plant's visual appearance and therefore to its economic value.

The control of height and branching of many ornamental plants represents a major challenge in commercial greenhouse production. Extremely tall and branched out pot plants are prone to breakage and increase production costs. Usually, in the production of ornamentals, stem growth promotion is not desired, with some exceptions, such as the cultivation of cut flowers (Zhang & Runkle, 2019). Chemical agents, such as plant growth retardants or gibberellic acid, are commonly used to regulate stem length; however, due to the potential negative effects of plant growth retardants on human health and the environment, limitation of their use is desirable (Rajapakse et al., 1999).

The development of plant vegetative architecture, including stem elongation and branching, is regulated mostly by phytochromes, although the participation of cryptochromes in inhibition of stem elongation has been documented (Liscum et al., 1992). Most plants show some degree of shade-avoidance response in an attempt to reach more photosynthetic light. Generally, under a reduced R : FR ratio, plants elongate. For example, in *Digitalis purpurea*' Dalmatian Peach' seedlings, the addition of 7.9 to 68.8 μmol m^{-2} s^{-1} FR light to white light ended in a 38 % increase in shoot length in a dose-dependent manner (Elkins et al., 2019).

EOD lighting has been widely used recently to regulate extension growth by manipulating the R : FR ratio (Downs & Thomas, 1982; Islam et al., 2015; Ilias & Rajapakse, 2005). For example, Islam et al. (2014) exposed two poinsettia cultivars to R or FR EOD lighting for 30 min at 10 μmol m^{-2} s^{-1} after a 10-hour short day (SD). After 11 weeks, the internode length was 55 % to 107 % greater under EOD-FR compared with EOD-R.

Many studies illustrate the variability in species responses to light quality and underscore the complexity of how light regulates stem extension and (flowering) in plants (Craig & Runkle, 2013). Variation in plant height was found in two poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch) cultivars under the 2-h EOD lighting treatments, regardless of light quality. The effect was attributed to different cultivar specificity (Zhang & Runkle, 2019) and the results were in accordance with the findings of Rajapakse et al. (1993) (cited by Zhang & Runkle, 2019) who reports that same EOD-FR treatment increased height in one chrysanthemum cultivar by ≈ 50 % while the plant height of the other cultivar was similar to control. Different sensitivity to the R : FR ratio in stem elongation was observed between three SDP species (*Chrysanthemum* x *morifo-*

lium (Ramat.) Hemsl.) , *Dahlia* x *hortensis* A. Guillaumin, and *Tagetes erecta* L.) (Craig & Runkle, 2013), as well as between five LDP species (*Campanula carpatica* Jacq., *Coreopsis* × *grandiflora* Hogg ex Sweet, *Lobelia* × *speciosa* Sweet, *Pisum sativum* L., and *Viola* × *wittrockiana* Gams) (Runkle & Heins, 2001). In the latter study, reaction to lower R : FR ratio was greater in the sun-adapted species *C. carpatica* and *Platycodon grandiflorus* (Jacq.) A.DC.. The extension growth was promoted in all five species under B-deficient environment. Based on the results, the authors suggested that blue light plays an important role in mediating stem extension in LDP. Similar conclusions were drawn from the study of petunia (*Petunia x hybrida* hort. ex E.Vilm.,), geranium (*Pelargonium x hortorum* L.H.Bailey) and coleus (*Solenostemon scutellaroides* (L.) Codd) seedlings grown under different combinations of R, FR, and B light at various intensities in a 16-hour photoperiod. The height of the plants increased linearly with lowering R : FR ratio and moderately high B light attenuated the effects of the R : FR ratio on extension growth (Park & Runkle, 2019).

For plants marketed for their flowers, the most crucial point is coordinated control of time and duration of flowering. One of the strategies to manage flowering without using chemicals is light quality manipulation, particularly the R : FR ratio. Low-intensity photoperiodic lighting is usually used by horticulturists to alter the natural photoperiod to manipulate flowering. The EOD lighting of different duration has been used to extend the natural photoperiod and create long days; recently, the feasibility of adjusting spectral quality of EOD supplemental lighting has broadened the range of possible applications. Another favourable approach of lighting, used for flowering control, is the use of night interruption (NI) lighting, using the light of differing spectral quality. The advantage of short-term lighting is its economic feasibility as a result of its lower intensity requirements and shorter application duration (Chia & Kubota, 2010).

Photoperiodic flowering in plants is achieved by the interactions of genes involved in the developmental control of floral initiation, the regulation of the circadian clock, and the signal transduction of phytochromes and cryptochromes receptors. For successful planning of flowering, coordination of all environmental factors, including light quality and intensity, timing, temperature, etc., has to be considered.

In SDP, it is acknowledged that R light is most effective at inhibiting flowering (Craig & Runkle, 2013), but the sensitivity to the R : FR ratio and the duration of NI or EOD lighting differs between species or even cultivars.

In three SDP ornamental species chrysanthemum , dahlia , and African marigold flowering was delayed at R : FR 0.66 or greater. NI treatment that primarily emits R light was determined to be the most effective in terms of flower inhibition for the SDP studied. The comparison between incandescent lights that emit low levels of B light and LED lighting without B light were shown to have similar effects on flowering, and it was found that B light, not FR light alone, was not needed to regulate flowering (Craig & Runkle, 2013).

In LDP, flowering is promoted most when artificial lighting contains R and FR light, creating a moderately low Pfr/Pt, compared to light deficient in FR (high Pfr/Pt) (Runkle & Heins, 2001). To determine the effect of FR, R, and B light on flowering in five LDP species (*Campanula carpatica* Jacq., *Coreopsis* × *grandiflora* Hogg ex Sweet, *Lobelia* × *speciosa* Sweet, *Pisum sativum* L., and *Viola* × *wittrockiana* Gams), plants were exposed to 16-hour photoperiod under R-, FR- and B-deficient photoselective filters in addition to a control (no filter). FR light promoted flowering, although the analysed species showed different sensitivity to changed R : FR ratios. Reaction to lower R : FR ratio was, similarly as in stem elongation, greater in sun-adapted species *C. carpatica* and *C. x grandiflora.* The authors observed that the time of day when the plants are exposed to FR could also have an impact on flowering. In this study, B light had little or no effect on flowering, but since the absorption of B light has been shown to accelerate flowering in *Arabidopsis* and *Hyoscyamus*, the authors speculated that the intensity of B light was to low to induce the effect. The promotion of flowering by 7-11 days at low R : FR ratio was also observed in LDP petunia (Park & Runkle, 2019). As in previously cited studies, authors found that B light had no effect on on the FR light promotion of flowering.

Zahedi and Sarikhani (2016) studied the simultaneous effects of EOD FR treatment duration, temperature, and plant age on strawberry (*Fragaria x ananassa* Duchesne) development. They demonstrated their synergistic effect on the morphological traits and flower induction.

4.4 FR IN PLANT DEFENCE AND INTERACTIONS WITH ABIOTIC FACTORS

A vast number of studies has revealed the importance of phytochromes in plant development, although more recently, the evidence of their role in modulating different biotic and abiotic stresses is expanding. The understanding of the molecular mechanisms underlying the reactions controlled by phytochrome is constantly increasing. This and the availability of phytochrome mutants in different plant species facilitates further investigation of their role in the complex coordination of responses to various environmental factors. The proportion of R and FR light detected by phytochromes, especially phy B, has been shown to play a role in regulating signalling induced by herbivory (Ballaré, 2017), salt stress (Cao et al., 2018), drought stress (González et al., 2012) and temperature change (Lorenzo et al., 2016). Because those environmental conditions can greatly affect plant productivity, the studies on phytochrome regulation of stress responses have become a hot spot of research.

To survive and prosper in different natural conditions, plants must precisely allocate their limited resources between growth and defence. In an environment with low R : FR ratios (shade) the numerous shade avoidance responses are triggered; however, the plant's immunity is reduced (McGuire & Agrawal, 2005; Roberts et al., 2006). The main mechanism underlying defence suppression is the simultaneous downregulation of jasmonate and salicylic acid signalling by low ratios of R : FR radiation (De Wit et al., 2013; Ballaré, 2014). Exposure to a low R : FR light reduced plant resistance against Botrytis cinerea Pers. in Arabidopsis thaliana (L.) Heynh., (Cerrudo et al., 2012; Cargnel et al., 2014) and in tomatoes (Ji et al., 2019). It was shown that fluorescent illumination with a high R : FR ratio (7.0) in comparison with low R : FR ratio (1.1) improved the resistance of cucumber seedlings to powdery mildew (Shibuya et al., 2011).

Several studies demonstrated that phytochromes, especially phyB, play a role in modulating signalling induced by drought stress. Drought tolerance can be promoted or depressed, according to species (Demotes-Mainard, 2016). Gonzalez et al. (2012) evaluated how phyB modulates drought-tolerance response by comparing wild-type *Arabidopsis thaliana* adult plants to the null phy B mutant in response to water shortage. They proposed that phyB, according to changes in the R : FR ratio, increases drought tolerance by enhancing ABA sensitivity. Drought tolerance could also be affected by morphological responses to the R : FR ratio, such as changes in leaf area and hyponasty (Demotes-Mainard et al., 2016).

Recently, it has been shown that exposure to lower R : FR ratios improve tomato tolerance to salt stress most likely through regulation of antioxidant enzymes and non-enzymatic systems and that phytochrome B1 plays a very important role in this process. The improved salt stress tolerance was accompanied by a higher actual quantum yield of photosynthesis (ΦPSII), electron transport rate (ETR), and photochemical quenching (qP), indicating overall healthier growth (Cao et al., 2018).

5 CONCLUSION

The growing knowledge about photoreceptors and the mechanisms by which light controls plant growth and development have been successfully applied in horticulture. In recent years, light-emitting diodes (LEDs) have facilitated the control of the spectral composition of supplemental lighting and expanded the application of different solo or combined wavelengths in greenhouse plant production. Adding FR light or lowering R : FR ratio has been shown to positively affect the photosynthesis and resulting biomass of some vegetable species. FR light can simultaneously elicit different morphological and physiological responses, among which only some are desirable. To efficiently use lighting for the manipulation of desired characteristics and to provide the horticultural products of high quality, more research is needed to understand better the mutual influence of different spectral combinations of light and other environmental conditions present at cultivation (e.g., photoperiod, temperature, water availability, nutrition, etc.). In many cases, it has been shown that the photomorphogenic responses are species-specific and, since the majority of research is done on a few model species, the analyses of other species would be necessary.

6 REFERENCES

Ahmad, M., Jarillo, J.A., Smirnova, O., & Cashmore, A.R. (1998). The CRY1 blue light photoreceptor of *Arabidopsis* interacts with phytochrome A in vitro. *Molecular Cell*, *1*, 939–948. https://doi.org/10.1016/s1097-2765(00)80094-5

Akutsu, M., Izena, J., & Takakura, T. (2017). Effect of EOD-FR Treatment on the growth and morphology of Japanese Mustard Spinach and Pak-choi. *Horticultural Research (Japan)*, *16*(4), 449-454. https://doi.org/10.2503/hrj.16.449

Alrifai, O., Hao, X., Marcone, M.F., & Tsao, R. (2019). Current review of the modulatory effects of LED lights on photosynthesis of secondary metabolites and future perspectives of microgreen vegetables. *Journal of Agricultural and Food Chem*istry, *67*, 6075-6090. http://dx.doi.org/10.1021/acs.jafc.9b00819

Ballaré, C. L. (2017). Phytochrome Responses: Think Globally, Act Locally. *Trends in Plant Science*, *22*(11), 909-911. https://doi.org/10.1016/j.tplants.2017.09.004

Ballaré, C.L. (2014). Light regulation of plant defense. *Annual Review of Plant Biology*, *65*, 335–363. https://doi.org/10.1146/annurev-arplant-050213-040145

Bantis, F., Smirnakou, S., Ouzounis, T., Koukounaras, A., Ntagkas, N., & Radoglou, K. (2018). Current status and recent achievements in the field of horticulture with the use of light-emitting diodes (LEDs). *Scientia Horticulturae*, *235*, 437-451. https://doi.org/10.1016/j.scienta.2018.02.058

Bilodeau, S.E., Wu, B., Rufyikiri, A., MacPherson, S., & Lefsrud, M. (2019). An update on plant photobiology and implications for *Cannabis* production. *Frontiers in Plant Science*, *10*, 296. https://doi.org/10.3389/fpls.2019.00296

Bradbeer, J.W. (1971). Plastid Development in Primary Leaves of Phaseolus vulgaris: the effect of short blue, red, far-red, and white light treatments on dark-grown plants. Journal of Experimental Botany, *22*(2), 382–390. https://doi.org/10.1093/jxb/22.2.382

Cao, K., Yu, J., Xu, D., Ai, K., Bao, E., & Zou, Z. (2018). Exposure to lower red to far-red light ratios improve tomato tolerance to salt stress. *BMC Plant Biology*, *18*, 92. https://doi.org/10.1186/s12870-018-1310-9

Cargnel, M.D., Demkura, P.V., & Ballaré, C.L. (2014). Linking phytochrome to plant im-munity: low red: far-red ratios increase Arabidopsis susceptibility to *Botrytis cinerea* by reducing the biosynthesis of indolic glucosinolates and camalexin. *New Phytologist, 204*, 342–354. https://doi.org/10.1111/nph.13032

Casal, J.J., & Smith, H. (1989). The function, action and adaptive significance of phytochrome in light-grown plants. *Plant, Cell & Environment, 12*, 855-862. https://doi.org/10.1111/j.1365-3040.1989.tb01966.x

Casal, J.J., Sanchez, R.A., & Yanovsky, M.J. (1997). The function of phytochrome A. *Plant, Cell & Environment*, *20*(6), 313-819. https://doi.org/10.1046/j.1365-3040.1997.d01-113.x

Cerrudo, I., Keller, M.M., Cargnel, M.D., et al. (2012). Low red/far-red ratios reduce *Arabidopsis* resistance to *Botrytis cinerea* and jasmonate responses via a COI1-JAZ10-dependent, salicylic acid-independent mechanism. *Plant Physiology, 158*, 2042–2052. https://doi.org/10.1104/pp.112.193359

Chang, M.-H., Das, D., Varde, P., & Pecht, M. (2012). Light emitting diodes reliability review. *Microelectronics Reliability, 52*, 762–782. https://doi.org/10.1016/j.microrel.2011.07.063

Chia, P., & Kubota, C. (2010). End-of-day far-red light quality and dose requirements for tomato rootstock hypocotyl elongation. *HortScience*, *45*(19), 1501-1506. https://doi.org/10.21273/HORTSCI.45.10.1501

Cho, J., Park, J. H., Kim, J. K., & Schubert, E. F. (2017). White light-emitting diodes: history, progress, and future. *Laser & Photonics Reviews, 11*, 1600147. https://doi.org/10.1002/lpor.201600147

Craig, D.S., & Runkle, E.S. (2013). A Moderate to high red to far-red light ratio from light-emitting diodes controls flowering of short-day plants. *Journal of the American Socety for Horticultural Science*, *13*(3), 167-172. https://doi.org/10.21273/JASHS.138.3.167

De Simone, S., Oka, Y., Inoue, Y., Nishioka, N., Tadano, S., & Inoue, Y. (2000). Evidence of phytochrome mediation in the low-pH-induced root hair formation process in lettuce (*Lactuca sativa* L. cv. Grand Rapids) seedlings. *Journal of Plant Research*, *113*, 45-53. https://doi.org/10.1007/PL00013915

De Wit, M., Spoel, S.H., Sanchez-Perez, G.F., et al. (2013). Perception of low red: far-red ratio compromises both salicylic acid- and jasmonic acid-dependent pathogen de-fences in *Arabidopsis*. *The Plant Journal, 75*, 90–103. https://doi.org/10.1111/tpj.12203

Demotes-Mainard, S., Péron, T., Corot, A. et al. (2016). Plant responses to red and far-red lights, applications in horticulture. *Environmental and Experimental Botany*, *121*, 4-21. https://doi.org/10.1016/j.envexpbot.2015.05.010

Downs, R.J., & Thomas, J.F. (1982) Phytochrome regulation of flowering in the long-day plant, *Hyoscyamus niger*. *Plant Physiology*, *70*(3), 898-900. https://doi.org/10.1104/pp.70.3.898

Elkins, C.A., Martin, M., & van Iersel, M.W. (2019). Growth and morphological responsesof Digitalis and Rudbeckia seedlings to supplemental far-red LED light. *HortScience*, *54*(9) Supplement, S89.

Fanwoua, J., Vercambre, G., Buck-Sorlin, G., Dieleman, J.A., de Visser, P., & Génard, P. (2019). Supplemental LED lighting affects the dynamics of tomato fruit growth and composition. *Scientia Horticulturae*, *256*, 109402. https://doi.org/10.1016/j.scienta.2019.108571

Favre, N., Bárcena, A., Bahima, J.V., Martinez, G., & Costa., L. (2018). Pulses of low intensity light as promising technology to delay postharvest senescence of broccoli. *Postharvest Biology and Technology*, *142*, 107-114. http://dx.doi.org/10.1016/j.postharvbio.2018.01.009

Franklin, K.A., & Quail, P.H. (2010). Phytochrome functions in *Arabidopsis* development. *Journal of Experimental Botany*, *61*(1), 11-24. https://doi.org/10.1093/jxb/erp304

Furuya, M. (1993). Phytochromes: their molecular species, gene families and functions. *Annual Review of Plant Physiology and Plant Molecular Biology*, *44*, 617–645. https://doi.org/10.1146/annurev.pp.44.060193.003153

González, C.V., Ibbara, S.E., Piccoli, P.N., Botto, J.F., & Boccalandro H.E. (2012). Phytochrome B increases drought tolerance by enhancing ABA sensitivity in *Arabidopsis thaliana*. *Plant, Cell & Environment, 35*, 1958-1968. https://doi.org/10.1111/j.1365-3040.2012.02529.x

Gundel, P. E., Pierik, R., Mommer, L., & Ballaré, C. L. (2014). Competing neighbors: light perception and root function. *Oecologia*, *176*, 1–10. https://doi.org/10.1007/s00442-014-2983-x

Hao, X., Zheng, J., Little, C., & Khosla S. (2012). Led inter-lighting in year-round greenhouse mini-cucumber production. *Acta Horticulturae*, *956*, 335-340. https://doi.org/10.17660/ActaHortic.2012.956.38

Holmes, M.G., & Smith, H. (1977). Function of phytochrome in natural environment 1, Characterization of daylight for studies in photomorphogenesis and photoperiodism. *Photochemistry and Photobiology*, *25*, 533-538. https://doi.org/10.1111/j.1751-1097.1977.tb09124.x

Ilias, I.F., & Rajapakse, N. (2005). The effects of end-of-the-day red and far-red light on growth and flowering of *Petunia x hybrida* 'Countdown Burgundy' grown under photoselective films. *HortScience*, *40*(1), 131-133. http://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=16437346

Islam, M.A., Gislerod, H.R., Torre, S., & Olsen, J.E. (2015). Control of shoot elongation and hormone physiologyin poinsettia by light quality provided by light emitting diodes – a minireview. *Acta Horticulturae*, *1104*, 131-136. https://doi.org/10.17660/ActaHortic.2015.1104.20

Islam, M.A., Tarkowska, D., Clarke, J.L., Blystad, D.R., Gislerod, H.R., Torre, S., & Olsen, J.E. (2014). Impact of end-of-day red and far-red light on plant morphology and hormone physiology of poinsettia. *Scientia Horticulturae*, *174*, 77-86. https://doi.org/10.1016/j.scienta.2014.05.013

Ji, Y., Ouzounis, T., Courbier, S. et al. (2019). Far-red radiation increases dry mass partitioning to fruits but reduces *Bot-*

rytis cinerea resistance in tomato. *Environmental and Experimental Botany*, *168*, 103889. https://doi.org/10.1016/j.envexpbot.2019.103889

Jung, J.H. (2016). Phytochromes function as thermosensors in *Arabidopsis*. *Science*, *354*, 886-889. https://doi.org/10.1126/science.aaf6005

Kalaitzoglou, P., van Ieperen, W., Harbinson, J., van der Meer, M., Martinakos, S., Weerheim, K., Nicole, C.C.S., & Marcelis, F.M. (2019). Effects of continuos or end-of-day far-red light on tomato plant growth, morphology, light absorbtion, and fruit production. *Frontiers in Plant Science*, *10*, 322. https://doi.org/10.3389/fpls.2019.00322

Kang, C., Lian, H., Wang, F., Huang, J., & Yang, H. (2009). Cryptochromes, Phytochromes, and COP1 Regulate Light-Controlled Stomatal Development in Arabidopsis. Plant Cell, 21, 2624-2641. https://doi.org/10.1105/tpc.109.069765

Kim, H., Lin, M., & Mitchell, C.A. (2019). Light spectral and thermal properties govern biomass allocation in tomato through morphological and physiological changes. *Environmental and Experimental Botany*, *157*, 228-240. https://doi.org/10.1016/j.envexpbot.2018.10.019

Klem, K., Gargallo-Garriga A., Rattanapichai, W., Oravec, M., Holub, P., Veselá, B., Sardans, J., Peñuelas, J., & Urban, O. (2019). Distinct morphological, physiological, and biological responses ti light quality in barley leaves and roots. *Frontiers in Plant Science*, *10*(1026), 1-18. https://doi.org/10.3389/fpls.2019.01026

Kono, M., Kawaguchi, H., Mizusawa, N.,Yamori, W., Suzuki , Y., & Terashima, I. (2020). Far-Red Light Accelerates Photosynthesis in the Low-Light Phases of Fluctuating Light. Plant and Cell Physiology, *61*(1), 192–202. https://doi.org/10.1093/pcp/pcz191

Kopsell, D.A., & Sams, C.E. (2013). Increases in shoot tissue pigments, glucosinolates, and mineral elements in sprouting broccoli after exposure to short-duration blue light from light emitting diodes. *Journal of the American Socety for Horticultural Science*, *138*, 31-37. https://doi.org/10.21273/JASHS.138.1.31

Kurepin, L.V. Emery, R.J., Pharis, R.P., & Reid, D.M. (2007). Uncoupling light quality from light irradiance effects in *Helianthus annuus* shoots. Putative roles for plant hormones in leaf and internode growth. *Journal of Experimental Botany*, *58*, 2145-2157. https://doi.org/10.1093/jxb/erm068

Lagarias, J.C., & Rapoport, H. (1980). Chromopeptides from phytochrome: the structure and linkage of the Pfr form of the phytochrome chromophore. *Journal of the American Chemical Society*, *102*, 4821-4828. https://doi.org/10.1021/ja00534a042

Larcher, W. (2003). *Physiological plant ecology*. Berlin Heidelberg: Springer-Verlag.

Legris, M., Ince, Y.Ç., & Fankhauser, C. (2019). Molecular mechanisms underlying phytochrome-controlled morphogenesis in plants. *Nature Communications, 10*, 5219. https://doi.org/10.1038/s41467-019-13045-0

Li, Q., & Kubota, C. (2009). Effects of supplemental light quality on growth and phytochemicals of baby leaf lettuce. *Environmental and Experimental Botany, 67*, 59- 64. https://doi.org/10.1016/j.envexpbot.2009.06.011

Liscum, E.; Young, J.C.; Poff, K.L., & Hangarter, R.P. (1992). Genetic separation of phototropism ad blue light inhibition of stem elongation. *Plant Physiology*, *100*, 267-271. https://dx.doi.org/10.1104%2Fpp.100.1.267

Lorenzo, C.D., Sanchez-Lamas, M., Antonietti, M.S., & Cerdan, P.D. (2016). Emerging hubs in plant light and temperature signaling. *Photochemistry and Photobiology 92*, 3–13. https://doi.org/10.1111/php.12535

Más, P., Devlin, P.F., Panda, S., & Kay, S.A. (2000). Functional interaction of phytochrome B and cryptochrome 2. *Nature*, *408*, 207–211. https://doi.org/10.1038/35041583

Massa, G. D., Emmerich, J. C., Mick, M. E., Kennedy, R., Morrow, R. C., & Mitchell, C. A. (2005). Development and testing of an efficient LED intracanopy lighting design for minimizing Equivalent System Mass in an advanced life-support system. *Gravitational and Space Biology Bulletin*, *18,* 87–88. http://gravitationalandspacebiology.org/index.php/journal/article/viewFile/351/354

Massa, G.D., Kim, H.-H., Wheeler, R.M., & Mitchell, C.A. (2008). Plant productivity in response to LED lighting. *HortScience*, *43*, 1951–1956. https://doi.org/10.21273/HORTSCI.43.7.1951

McCree, K.J. (1972). The action spectrum, absorptance and quantum yield of photosynthesis in crop plants. *Agricultural Meteorology*, *9*, 191–216. https://doi.org/10.1016/0002-1571(71)90022-7

McGuire, R., & Agrawal, A.A. (2005). Trade-offs between the shade-avoidance response and plant resistance to herbivores? Tests with mutant *Cucumis sativus*. *Functional Ecology*, *19*, 1025–1031. https://doi.org/10.1111/j.1365-2435.2005.01047.x

Meng, Q., & Runkle, E.S. (2019). Far-red radiation interacts with relative and absolute blue and red photon flux densities to regulate growth, morphology, and pigmentation of lettuce and basil seedlings. *Scientia Horticulturae*, *255*, 269-280. https://doi.org/10.1016/j.scienta.2019.05.030

Meng, Q., Kelly, N., & Runkle, E.S. (2019). Substituting green or far-red radiation for blue radiation induces shade avoidance and promotes growth in lettuce and kale. *Environmental and Experimental Botany*, *162*, 383-391. https://doi.org/10.1016/j.envexpbot.2019.03.016

Morrow, R.C. (2008). LED lighting in horticulture. *HortScience, 43*, 1947-1950.

Myers, J. (1971). Enhancement studies in photosynthesis. *Annual Review of Plant Physiology*, *22*, 289–312. https://doi.org/10.1146/annurev.pp.22.060171.001445

Nagata, M., Yamamoto, N., Shigeyama, T. et al. (2015). Red/far red light controls arbuscular mycorrhizal colonization via jasmonic acid and strigolactone signalling. *Plant and Cell Physiology*, *56*(11), 2100-2109. https://doi.org/10.1093/pcp/pcv135

Nájera, C., Guil-Guerrero, J.L., Enriquez, L.J., & Álvaro, J.E. (2018). LED-enhanced dietary and organoleptic qualities in postharvest tomato fruit. *Postharvest Biology and Technology*, *145,* 151-156. https://doi.org/10.1016/j.postharvbio.2018.07.008

Nelson, J. A., & Bugbee, B. (2014). Economic analysis of greenhouse lighting: light emitting diodes vs. high intensity discharge fixtures. *PLOS ONE*, *9*(6), e99010. https://doi.org/10.1371/journal.pone.0099010

Nishimura, Y., Wada, E., Fukumoto, Y., Aruga, H., & Shimoi, Y. (2012). The effect of spectrum conversion covering film on cucumber in soilless culture. *Acta Horticulturae*, *956*, 481-487. https://doi.org/10.17660/ActaHortic.2012.956.56

Olle, M., & Viršile, A. (2013). The effects of light-emitting diode lighting on greenhouse plant growth and quality. *Agricultural and Food Science, 22*, 223–234. https://doi.org/10.23986/afsci.7897

Park, Y., & Runkle, E.S. (2017). Far-red radiation promotes growth of seedlings by increasing leaf expansion and whole-plant net assimilation. *Environmental and Experimental Botany*, *136*, 41-49. https://doi.org/10.1016/j.envexpbot.2016.12.013

Park, Y., & Runkle, E.S. (2019). Blue radiation attenuates the effects of the red to far-red ratio on extension growth but not on flowering. *Environmental and Experimental Botany*, *168*, 103871. https://doi.org/10.1016/j.envexpbot.2019.103871

Parks, B.M., Quail, P.H., & Hangarter, R.P. (1996). Phytochrome A regulates red light induction of phototropic enhancement in *Arabidopsis*. *Plant Physiology*, *110*, 155–162.

Patel, D., Basu, M., Hayes, S., Majlath, I., Hetherington, F.M., Tschaplinski, T.J., & Franklin, K.A. (2013). Temperature-dependent shade avoidance involves the receptor-like kinase ERECTA. *The Plant Journal*, *73*, 980–992. https://doi.org/10.1111/tpj.12088

Pinho, P., Jokinen, K., & Halonen, L. (2017). The influence of the LED light spectrum on the growth and nutrient uptake of hydroponically grown lettuce. *Lighting Reearch & Technology*, *49*(7), 866-881. https://doi.org/10.1177%2F1477153516642269

Rajapakse, N.C., Young, R.E., McMahon, M.J., & Oi, R. (1999). Plant height control by photoselective filters: current status and future prospects. *Hort Technology*, *9*(4), 618-624. https://doi.org/10.21273/HORTTECH.9.4.618

Roberts, M.R., & Paul, N.D. (2006). Seduced by the dark side: integrating molecular and ecological perspectives on the influence of light on plant defence against pests and pathogens. *New Phytologist*, *170*, 677–699. https://doi.org/10.1111/j.1469-8137.2006.01707.x

Runkle, E. S. (2013). Manipulating light quality to elicit desirable plant growth and flowering responses. *IFAC Proceedings Volumes*, *46*(4), 196-200. https://doi.org/10.3182/20130327-3-JP-3017.00044

Runkle, E.S., & Heins, R.D. (2001). Specific Functions of red, far red, and blue light in flowering and stem extension of long-day plants. *Journal of the American Socety for Horticultural Science*, *126*(3), 275-282. https://doi.org/10.21273/JASHS.126.3.275

Sadras, V. O., Hall, A. J., Trapani, N., & Vilella, F. (1989). Dynamics of rooting and root-length: leaf-area relationships as affected by plant population in sunflower crops. *Field Crops Research*, *22*, 45–57. https://doi.org/10.1016/0378-4290(89)90088-9

Sánchez-Lamas, M., Lorenzo, C.D., & Cerdán, P.D. (2016). Bottom-up assembly of the phytochrome network. *PLOS Genetics*, *12* (11), e1006413. https://dx.doi.org/10.1371%2Fjournal.pgen.1006413

Shibuya, T., Endo, R., Kitaya, Y., & Hayashi, S. (2016). Growth analysis and photosynthesis measurements of cucumber seedlings grown under light with different red to far-red ratios. *HortScience*, *51*(7), 843-846. https://doi.org/10.21273/HORTSCI.51.7.843

Shibuya, T., Itagaki, K., Tojo, M., Endo, R., & Kitaya, Y. (2011). Fluorescent illumination with high red-to-far-red ratio improves resistance of cucumber seedlings to powdery mildew. *HortScience,* 46(3), 429-431. https://doi.org/10.21273/HORTSCI.46.3.429

Singh, D., Basu, C., Meinhardt-Wollweber, M., & Roth, B. (2015). LEDs for energy efficient greenhouse lighting. *Renewable and Sustainable Energy Reviews*, *49*, 139-147. https://doi.org/10.1016/j.rser.2015.04.117

Srikanth, A., & Schmid, M. (2011). Regulation of flowering time: all roads lead to Rome. *Cellular and Molecular Life Sciences*, *68*, 2013-2037. https://doi.org/10.1007/s00018-011-0673-y

Stutte, G.W., Edney, S., & Skerritt, T. (2009). Photoregulation of bioprotectant content of red leaf lettuce with light-emitting diodes. *HortScience*, *44*(1), 79-82. https://doi.org/10.21273/HORTSCI.44.1.79

Suzuki, A., Suriyagoda, L., Shigeyama, T. et al. (2011). *Lotus japonicus* nodulation is photomorphogenetically controlled by sensing the red/far red (R/FR) ratio through jasmonic acid (JA) signalling. *Proceedings of the National Academy of Sciences of the United States of America*, *108,* 16837-16842. http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1105892108/-/DCSupplemental

Taiz, L., & Zeiger, E. (2014). *Plant physiology*. Sunderland, MA: Sinauer Associates Inc.

Thiele, A., Herold, M., Lenk, I., Quail, P.H., & Gatz, C. (1999). Heterologous expression of *Arabidopsis* phytochrome B in transgenic potato influences photosynthetic performance and tuber development. *Plant Physiology*, *120*, 73-82. https://doi.org/10.1104/pp.120.1.73

Tokuhisa, J.G., Daniels, S.M., & Quail, P.H. (1985). Phytochrome in green tissue: Spectral and immunochemical evidence for two distinct molecular species of phytochrome in light-grown *Avena sativa* L. *Planta*, *164*, 321-332. https://doi.org/10.1007/BF00402943

Turnbull, M.H., & Yates, D.J. (1993). Seasonal variation in the red/far-red ratio and photon flux density in an Australian sub-tropical rainforest. Agricultural and Forest Meteorology, *64*(1-2), 111-127. https://doi.org/10.1016/0168-1923(93)90096-Z

Viczián, A., Klose, C., Adam, E., & Nagy, F. (2017). New insights of red light-induced development. *Plant, Cell & Environment*, *40*, 2457-2468. https://doi.org/10.1111/pce.12880

Viršilė, A., Olle, M., & Duchovskis, P. (2017). "LED lighting in horticulture" in *Light Emitting Diodes for Agriculture.* ed. S.D. Gupta. (Singapore: Springer), 113–147.

Yang, F., Feng, L., Liu, Q., et al. (2018). Effect of interactions between light intensity and red-to far-red ratio on the photosynthesis of soybean leaves under shade condition. *Environmental and Experimental Botany*, *150*, 79-87. https://doi.org/10.1016/j.envexpbot.2018.03.008

Yang, Z., Kubota, C., Chia, P., & Kacira, M. (2012). Effect of end-of-day far-red light from a movable LED fixture on squash rootstock hypocotyl elongation. *Scientia Horticulturae*, *136*, 81-86. https://doi.org/10.1016/j.scienta.2011.12.023

Yeh, N., & Chung, J.-P. (2009). High-brightness Leds-Energy efficient lighting sources and their potential in indoor plant cultivation. *Renewable and Sustainable Energy Reviews, 13,* 2175–2180. https://doi.org/10.1016/j.rser.2009.01.027

Zahedi, S.M., & Sarikhani, H. (2016). Effect of far-red light, temperature, and plant age on morphological changes and induction of flowering of a 'June-bearing' strawberry. *Horticulture, Environment and Biotechnology*, *57*(4), 340-347. https://doi.org/10.1007/s13580-016-0018-8

Zhang, M., & Runkle, E.S. (2019). Regulating flowering and extension growth of Poinsettia using red and far-red light-emitting diodes for end-of-day lighting. *HortScience*, *54*(2), 323-327. https://doi.org/10.21273/HORTSCI13630-18

Zhang, M., Wogyhitman, C.M., & Runkle, E.S. (2019a). Manipulating growth, colour, and taste attributes of fresh cut lettuce by greenhouse supplemental lighting. *Scientia Horticulturae*, *252*, 274-282. https://doi.org/10.1016/j.scienta.2019.03.051

Zhang, Y., Zhang, Yu., Yang, Q., & Tao, L. (2019b). Overhead supplemental far-red light stimulates tomato growth under intra-canopy lighting with LEDs. *Journal of Integrative Agricultre*, *17*, 62-69. https://doi.org/10.1016/S2095-3119(18)62130-6

Zhen, S., & Bugbee, B. (2019). Far-red photons are necessary for efficient photosynthesis: whole-canopy photosynthesis and radiation capture. *HortScience*, *54*(9), S178. https://doi.org/10.1111/pce.13730

Zhen, S., & van Iersel, M.W. (2017). Far-red light is needed for efficient photochemistry and photosynthesis. *Journal of Plant Physiology*, *209*, 115-122. https://doi.org/10.1016/j.jplph.2016.12.004

Zhen, S., Haidekker, M., & van Iersel, M.W. (2019). Far-red light enhances photochemical efficiency in a wavelength-dependent manner. *Physiologia plantarum*, *167*(1), 21-33. https://doi.org/10.1111/ppl.12834

Zou, J., Zhang, Y., Zhang, Yu., Bian, Z., Fanourakis, D., Yang, Q., & Li, T. (2019). Morphological and physiological properties of indoor cultivated lettuce in response to additional far-red light. *Scientia Horticulturae*, *257*, 108725. https://dx.doi.org/10.1016/j.scienta.2019.108725

doi:10.14720/aas.2020.116.1.1583 Original research article / izvirni znanstveni članek

Bioactivity and effects of spraying interval of selected plant extracts for control of pre-flowering insect pests of watermelon (*Citrulus lanatus*[Thunb.] Matsum. & Nakai)

Alao FATAI OLAITAN [1], Adebayo THIMOTY ABIODUN [1], Olaniran OLADELE ABIODUN [1, 2]

Received March 28, 2020; accepted July 31,2020.
Delo je prispelo 28. marca 2020, sprejeto 31. julija 2020.

Bioactivity and effects of spraying interval of selected plant extracts for control of pre-flowering insect pests of watermelon (*Citrulus lanatus*[Thunb.] Matsum. & Nakai)

Abstract: This experiment was conducted to determine the efficacy of plant extracts of *Tephrosial vogelii* Hook. f., *Moringa oleifera* Lam., *Annona squamosa* L. and cashew nut (*Anacardium occidentale* L.) at two intervals of spraying against major pre-flowering insect pests of watermelon. The results show that the effectiveness of each plant extracts depends on the interval of spraying. Plants sprayed at 7-day intervals had significantly less insect infestation when compared with that of 10-day interval of spraying. *T. vogelii* and *M. oleifera* had higher insecticidal efficacy than *A. squamosa* and cashew nut extracts but the applied plant extracts had the same insecticidal potential with Lambda-cyhalothrin against the observed insects during late planting season. Therefore, the plant extracts proved to be alternative to synthetic insecticide in the management of insect pests of watermelon at 7-day interval application sequence.

Key words: *Tephrosial vogelii*; *Moringa oleifera; Annona squamosa*: *Annacadium occidentale*; watermelon

Biološki učinki intervalov škropljenja z izvlečki izbranih rastlin za uravnavanje škodljivih žuželk vobdobju pred cvetenjem lubenice (*Citrulus lanatus*[Thunb.] Matsum. & Nakai)

Izvleček: Namen poskusa je bil določiti učinkovitosti izvlečka izbranih rastlin (*Tephrosial vogelii* Hook. f., *Moringa oleifera* Lam., *Annona squamosa* L. , *Anacardium occidentale* L.)v dveh intervalih škropljenja na glavne škodljivce lubenice v obdobju pred cvetenjem. Rezultati kažejo, da je učinkovitost vsakega od rastlinskih izvlečkov odvisna od interval škropljenja. Rastline, ki so bile škropljene v 7-dnevnih intervalih so bile značilno manj okužene v primerjavi s tistimi, ki so bile škropljene v 10-dnevnih intervalih. Vrsti *T. vogelii* in *M. oleifera* sta imeli večjo insekticidno učinkovitost kot vrsti *A. squamosa* in *Anacardium occidentale*. Uporabljeni rastlinski izvlečki so imeli na opazovane žuželke med pozno rastno sezono enak insekticidni potencial kot lambda-cihalotrin. Rastlinski izvlečki so se izkazali kot dobra alternativa sintetičnim insekticidom pri uravnavanju škodljivcev na lubenici pri 7-dnevnih intervalih uporabe.

Ključne besede: *Tephrosial vogelii; Moringa oleifera; Annona squamosa; Annacadium occidentale;* lubenica

---

[1] Ladoke Akintola University of Technology, Department of Crop and Environmental Protection, Ogbomoso, Oyo state, Nigeria
[2] Corresponding author, e-mail: oaolaniran@lautech.edu.ng

1 INTRODUCTION

Watermelon (*Citrullus lanatus*[Thunb.] Matsum. & Nakai) belongs to the Cucurbitaceae family, which includes about 118 genera and 825 species (Dane and Liu, 2007). It originates from Kalahari and Sahara deserts in Africa (Schippers, 2000) and now found in tropical and subtropical climates worldwide. However, this crop has been cultivated for a long time in Africa and in the middle East and Egypt (Huhet al., 2008; Gichimuet al., 2009).

Watermelon is a major cucurbit crop, which accounts for 6.8 % of the world area (second to tomato) devoted to vegetable production in 2005. A rough estimate of annual word value of watermelon exceeds $ 15 billion (FAO, 2005).Report claims that watermelon is the most popular cucurbits, followed by cucumber and melon (FAO, 2006).In Nigeria, it is grown intensively in the northern region and bulk of it is produced from this agro-ecological zone (IITA, 2007). Recently, its cultivation has spread to the west and east of southern Nigeria. But yields from this region are generally low due to insect pest infestation (Alao and Adebayo, 2015). Insect pests such as *Zonocerus variegatus* L., 1758, *Aulocophoral affini*s D., 1835, *Phyllotreta cruciferae* (Goeze, 1777), *Bactrocera cucurbitea* (C., 1849) e.t.c have been implicated to have caused economic damage to this crop (Olaifa, 1987; Alwood, 1999; Alao, 2015).

In view of the aforementioned fact, control of pests becomes imperative. The use of synthetic insecticides to control the menace of this problem is a common practice among the majority of farmers in developing countries. Most of the farmers are not aware of the hazardous effects of these synthetic insecticides due to lack of education. In the last decade, entomologists and environmentalists have proposed for the use of plant extracts as alternative to the synthetic insecticides. However, most of these plants are abundant in the tropical region and have also medicinal value (Isman, 2006). The use of plant extracts in the management of field and stored product pests in the developing countries is a welcome development in the pest management program.Insecticides derived from plants have been reported as the credible alternative to synthetic insecticides (Alao and Adebayo, 2011). Unlike conventional insecticides, plants derived insecticides contain an array of chemical compounds, which act concertedly on both behavioral and physiological processes.

Tephrosia vogelii Hook f. is a small leguminous shrub, which grows to a height of about three to four meters. It is a perennial shrub with flowers and pods. The plant is widely distributed in the tropical, sub-tropical and regions of the world (Al-Zahrani, 2007). *T. vogelii* is traditionally used for its inchthyotoxic, insecticidal and food parasitical properties (Ibrahimet al., 2000; Dzendaet al., 2007; Qureshiet al., 2010).*Moringa oleifera* Lam. is a pantropical treespecies that is known by such regional names as benzolive, drumstick tree, kelor, marango, saijhan and sajna (Fahey, 2005). It is the most widely cultivated species of a monogeneric family, the Moringaceae, that is native to the sub-Himalayan tracts of India and Pakistan. It has become naturalized in many locations in the tropics and is widely cultivated in Africa, Ceylon, Thailand, Burma, Singapore, West Indies, Sri Lanke India, Mexico, Malabar, Malaysia and the Philippines (Fashey, 2005; Shukla et al., 2011).The Annonaceae is a large family of almost exclusively tropical trees and shrubs comprising about 130 general and 2300 species (Cronguist, 1993). Leatemia and Isman (2004) reported that plant parts of some species of this family have been used traditionally as insecticides.The soursop (*Annona muricata* L.), sweetsop (*A. squamosa* L.), langsat (*Lansium domesticum* Corr.) and *Sandoricum koetijape* (Burm. f.) Merr. are abundant species in the tropics (Isman, 2006). Cashew (*Anacardium occidentale* L.) belongs to the family Anacardiaceae. The plant is a native to North-Eastern Brazil, where it is called by its Portuguese name caju (the fruit) or cajueiro (the tree) (Varghese and Pundir, 1964). Cashew nut extracts contains a high proportion of phenolic compounds, anarcadic acid and cardole (Oparaeke et al., 2005; Olotuah and Ofuya, 2010; Mukhopadyayet al,2010).

2 MATERIALS AND METHODS

2.1 STUDY SITE

The field experiment was conducted in the cropping season of 2011 and 2012 at Ladoke Akintola University of Technology (LAUTECH) Teaching and Research Farm, Ogbomoso, Oyo State, Nigeria.This region is position on longitude 4°3'E and latitude 10°5'N. The region climate is described as hot humid tropical falls in Southern Guinea Savannah of Nigeria with a mean temperature of 27 °C and annual rainfall of 1400mm, marked with dry and wet seasons, characterized by a bi-modal rainfall pattern with peaks in July and September and a short period of harmattan in between the seasons.

2.2 EXPERIMENTAL DESIGN AND MANAGEMENT

The experimental field was harrowed and ploughed once and thirty (30) plots were mapped out and each experimental plot size was 3 m x 3 m with four plant stand rows. This experiment was arranged and demarcated in a randomized complete block design. There were six treatments with three replicates. Two to four watermelon seeds (sugar baby) were sowed per hole. Two weeks after planting, thinning was done to one plant per stand. Each plot contained four plant rows.

2.3 PREPARATION OF PLANT EXTRACTS

The plants species screened for insecticidal properties were leaves of *T. vogelii, M. oleifera,* seeds of *A. squamosa* and cashew nuts. All the plant materials were air dried for 3-4 days to reduce the moisture content except cashew nut, which was left to stay for more than three weeks. In order to prepare extract from each of them, the target plant parts were crushed separately in a wooden mortal with pestle. 500 g each of the paste material was weighed separately with the sensitive scale into 10-litre plastic bucket containing 1000 ml of water. These were allowed to settle overnight and the aqueous suspensions were filtered through muslin cloth. The filtrate then served as stock solution for the experiment. 1000 ml were measured from the stock solution. This method of preparation of crude extracts is in line with already established method by Alao and Adebayo (2011), however, each of the plant extract and synthetic insecticide were further diluted with 1000 ml of water to achieve the same spraying volume for all the treatment.

2.4 TREATMENT APPLICATION

Application of plant extracts was done (i) as spray at 7-day intervals and five weekly observations were made (ii) or spray at 10-day intervals and four weekly observations were made. The synthetic insecticide lambda-cyhalothrin was applied once in a week at manufacture's recommended rate. The foliar application of the treatments was done early in the morning with hand-held sprayer of 2-litre capacity. Unsprayed plots were included.

2.5 DATA COLLECTION

Population densities of insect pests were counted visually from four plant stands in the two middle plant rows. The observed adult insects collected were taken to Insect Muzeum at University of Ibadan for the identification of the species.

Percentage of leaves defoliation: Estimation of rate of defoliated leaves were done 2nd week after spraying and the leaves showing evidence of shot holes were considered as being damaged by the studied insects. This was done visually by counting the defoliated leaves and total number of leaves from the four selected plant stands in the two middle plant rows. Percentage of defoliated leaves was determined using the formula described below:

$$\%\text{ leaves damaged} = \frac{\text{total no. of leaves produced per plant} - \text{no. of undamaged leaves}}{\text{total number of leaves produced}} \times 100$$

Yield: Three months after planting, the matured fruits were harvested and weighed on the field with manual scale in kilogram (kg), which was later calculated in tons per hectare (t ha^{-1}).

2.5 DATA ANALYSIS

Data collected were subjected to analysis of variance (ANOVA) using randomized complete block design. Significant means were separated with Duncan Multiple Range Test at 5 % probability. To normalize variances for all analyses, the numbers of insects were square-root transformed. However, insecticide efficacy was calculated using Abbott's formula:

$$\text{Efficacy} = (CA - TA / CA) \text{ x } 100.$$

CA – The total number of the insect from the untreated plants

TA – The total number of the insect from the treated plants

3 RESULTS

3.1 EFFECT OF INTERVAL OF SPRAYING OF INSECTICIDES ON *Aulocophora africana* Mon., 1855

During early planting season, there was no significant difference($p < 0.05$) in the efficacy of *M. oleifera* and *T. vogelii* at 7 days interval, but *T. vogelii* treated plots had significant lower($p < 0.05$)*A. africana* infestation than *M. oleifera* treated plots at 10 days interval. Meanwhile, the efficacy of *T. vogelii* was comparable with lambda-cyhalothrin at 7-day interval, but this observation contradicts what was observed at 10-day interval. During late planting, *M. oleifera* and *T. vogelii* had the same statistical effect ($p < 0.05$) on infestation at 7-day intervals, meanwhile *M. oleifera* significantly ($p < 0.05$) suppressed *A. africana* infestation as it was observed in lambda-cyhalothrin treated plots at 10-day interval. However, application of plant extracts at 7-day interval had significant effect ($p < 0.05$) on reduction *A. africana* infestation than that of 10-day interval (Table 1).

Table 1: Effect of interval of spraying of insecticides on Aulocophora africana population

		Spraying interval (days)		
	Insecticides	7	10	Efficacy (%)
Early Season	*Annona squamosa*	2.41 ± 0.12^{bc}	2.60 ± 0.18^{ab}	17.05
	Cashew nut	2.54 ± 0.15^{ab}	2.73 ± 0.14^{ab}	12.75
	Control	3.02 ± 0.15^{a}	3.02 ± 0.15^{a}	-
	Moringa oleifera	1.95 ± 0.12^{c}	2.48 ± 0.12^{abc}	26.65
	lambda-cyhalothrin	1.39 ± 0.26^{d}	1.39 ± 0.26^{d}	54.00
	Tephrosia vogelii	1.95 ± 0.12^{c}	2.33 ± 0.15^{bc}	29.15
	Spray interval (mean)	$2.21^{b} \pm 0.15$	$2.43^{a} \pm 0.17$	
Late Season	*Annona squamosa*	1.29 ± 0.24^{bc}	1.76 ± 0.19^{ab}	25.25
	Cashew nut	1.74 ± 0.23^{ab}	1.86 ± 0.17^{a}	11.75
	Control	2.04 ± 0.12^{a}	2.04 ± 0.12^{a}	-
	Moringa oleifera	0.71 ± 0.00^{d}	0.71 ± 0.00^{d}	100.00
	lambda-cyhalothrin	0.71 ± 0.00^{d}	0.71 ± 0.00^{d}	100.00
	Tephrosia vogelii	0.71 ± 0.00^{d}	1.17 ± 0.22^{cd}	71.35
	Spray interval (mean)	$1.20^{a} \pm 0.09$	$1.37^{b} \pm 0.12$	

Means with the same superscript(s) along the column are not significantly different at 5 % probability using DMRT.

3.2 EFFECT OF INTERVAL SPRAYING OF INSECTICIDES ON *COCCINELLA Septempunctata* Lin., 1758

Among the plant extracts, cashew nut extract had the least insecticidal effect on *C. occidentallis* at 7 and 10-day intervals during early planting season. None of the plant extracts showed comparable effect with lambda-cyhalothrin during early planting season. *T. vogelii* extract had the highest insecticidal potential when compared with other tested plant extract in the early planting season.

During late planting season, the tested plant extracts compete effectively with lambda-cyhalothrin at 7-day interval of spraying. This result is similar to what was observed at 10-day interval spraying except cashew nut extracts which had the highest *C. occidentalis* infestation among the treated plots (Table 2).

3.3 EFFECT OF INTERVAL OF SPRAYING OF INSECTICIDES ON *Monoleptas australis* Dej., 1836.

Application of *M. oleifera* had highest insecticidal efficacy (29.2 %), when compared with other plant extracts at 7-day interval of spraying during early planting season, but there were no significant difference ($p < 0.05$) between the efficacy of *M. oleifera* and *T. vogelii* at 10-day intervals. At 10-day interval cashew nut treatment had the highest population density of *Monolepta* spp. when compared with other plant extracts treated plants during early planting season. During late planting season, there was no significant difference ($p < 0.05$) between treated plots and untreated plots.

3.4 EFFECT OF SPRAYING INTERVALS ON RATE OF LEAF DEFOLIATION

The results obtained showed that unprotected leaves were heavily attacked by the observed insects during both seasons, but the intensity of defoliation was higher in the early planting season than late planting season. During early planting season, plants sprayed with *T. vogelii* and *M. oleifera* at 7 and 10 days–intervals had significantly better ($p < 0.05$) protected leaves against the leaf feeding insects, when compared with other plant extracts.

3.5 EFFECT OF SPRAYING INTERVALS ON YIELD (t ha^{-1})

Among the plant extracts applied, *T. vogelii* treated

Table 2: Effect of interval of spraying of insecticides on *Coccinela septempunctata* population

		Spraying interval (days)		
	Insecticides	7	10	Efficacy (%)
Early Season	*Annona squamosa*	2.26 ± 0.17^{bcd}	2.79 ± 0.15^{ab}	16.40
	Cashew nut	2.55 ± 0.00^{abc}	2.79 ± 0.15^{ab}	11.60
	Control	3.02 ± 0.15^{a}	3.02 ± 0.15^{a}	-
	Moringa oleifera	2.12 ± 0.00^{cd}	2.41 ± 0.12^{bcd}	25.00
	Lambda-cyhalothrin	1.39 ± 0.26^{e}	1.39 ± 0.26^{e}	54.00
	Tephrosia vogelii	1.95 ± 0.12^{d}	2.48 ± 0.12^{abcd}	26.66
	Spray interval (mean)	2.22^{b} ±0.12	2.48^{a} ±0.16	
Late Season	*Annona squamosa*	0.71 ± 0.00^{b}	0.90 ± 0.24^{b}	73.20
	Cashew nut	0.88 ± 0.15^{b}	1.27 ± 0.25^{ab}	36.00
	Control	1.68 ± 0.14^{a}	1.68 ± 0.14^{a}	-
	Moringa oleifera	0.71 ± 0.00^{b}	0.88 ± 0.15^{b}	73.80
	Lambda-cyhalothrin	0.71 ± 0.00^{b}	0.71 ± 0.00^{b}	100.00
	Tephrosia vogelii	0.71 ± 0.00^{b}	0.90 ± 0.24^{b}	73.20
	Spray interval (mean)	0.90^{a} ±0.05	1.06^{a} ±0.17	

Means with the same superscript(s) along the column are not significantly different at 5 % probability using DMRT

Table 3: Effect of interval of spraying of insecticides on *Monolepta* spp. population

		Spraying interval (days)		
	Insecticides	7	10	Efficacy (%)
Early Season	*Annona squamosa*	2.55 ± 0.00^{abc}	2.97 ± 0.11^{abc}	18.35
	Cashew nut	2.73 ± 0.19^{abc}	3.12 ± 0.19^{ab}	13.45
	Control	3.38 ± 0.21^{a}	3.38 ± 0.21^{a}	-
	Moringa oleifera	2.18 ± 0.19^{cd}	2.61 ± 0.11^{abc}	29.15
	Lambda-cyhalothrin	1.65 ± 0.30^{d}	1.65 ± 0.30^{d}	51.20
	Tephrosia vogelii	2.41 ± 0.17^{bcd}	2.65 ± 0.22^{abc}	25.15
	Spray interval (mean)	2.48^{b} ±0.18	2.73^{a} ±0.19	
Late Season	*Annona squamosa*	0.71 ± 0.00^{a}	0.71 ± 0.00^{a}	100.00
	Cashew nut	0.71 ± 0.00^{a}	0.71 ± 0.00^{a}	100.00
	Control	0.99 ± 0.24^{a}	0.99 ± 0.24^{a}	-
	Moringa oleifera	0.71 ± 0.00^{a}	0.71 ± 0.00^{a}	100.00
	Lambda-cyhalothrin	0.71 ± 0.00^{a}	0.71 ± 0.00^{a}	100.00
	Tephrosia vogelii	0.71 ± 0.00^{a}	0.71 ± 0.00^{a}	100.00
	Spray interval (mean)	0.76^{a} ± 0.04	0.76^{a} ± 0.04	

Means with the same superscript(s) along the column are not significantly different at 5 % probability using DMRT.

Table 4: Effect of interval of spraying of insecticides on rate of leaf defoliation (%)

		Spraying interval (days)		
	Insecticides	7	10	Insecticides (Mean)
Early Season	*Annona squamosa*	42.1 ± 0.75^{cd}	48.9 ± 0.61^{bc}	45.5^{bc}
	Cashew nut	46.9 ± 0.86^{bcd}	52.8 ± 0.62^{ab}	49.9^{b}
	Control	61.2 ± 0.65^{a}	61.2 ± 0.65^{a}	61.2^{a}
	Moringa oleifera	39.1 ± 0.81^{de}	45.0 ± 0.80^{bcd}	42.1^{c}
	Lambda-cyhalothrin	31.0 ± 0.65^{e}	31.0 ± 0.65^{e}	31.0^{d}
	Tephrosia vogelii	39.1 ± 0.81^{de}	45.0 ±0.80^{bcd}	42.1^{d}
	Spray interval (mean)	43.3^{b} ±0.76	47.3^{a} ±0.69	
Late Season	*Annona squamosa*	28.8 ± 0.65^{abcd}	37.2 ± 0.62^{ab}	33.0^{abc}
	Cashew nut	33.2 ± 0.00^{abc}	36.9 ± 1.02^{ab}	35.1^{bc}
	Control	43.1 ± 0.61^{a}	43.1 ± 0.61^{a}	43.1^{a}
	Moringa oleifera	23.9 ± 0.72^{bcd}	33.0 ± 0.84^{abc}	28.4^{bc}
	Lambda-cyhalothrin	15.0 ± 1.23^{d}	15.0 ± 1.23^{d}	15.0^{d}
	Tephrosia vogelii	17.7 ± 1.31^{cd}	28.8 ± 0.65^{abcd}	23.3^{d}
	Spray interval (mean)	26.9^{a} ±0.75	32.3^{a} ±0.83	

Means with the same superscript(s) along the column are not significantly different at 5 % probability using DMRT.

Table 5: Effect of interval of spraying of insecticides on melon yield (t ha^{-1})

		Spraying interval (days)		
	Insecticides	7	10	Yield(t ha^{-1})
Early Season	*Annona squamosa*	17.0 ± 0.79^{bcd}	13.3 ± 0.68^{bcd}	15.2^{b}
	Cashew nut	20.8 ± 0.94^{bc}	11.7 ± 0.72^{cd}	16.3^{b}
	Control	7.67 ± 0.53^{d}	7.67 ± 0.53^{d}	7.67^{c}
	Moringa oleifera	20.8 ± 0.94^{bc}	13.8 ± 0.61^{bcd}	17.3^{b}
	Lambda-cyhalothrin	30.7 ± 0.48^{a}	30.7 ± 0.48^{a}	30.7^{a}
	Tephrosia vogelii	22.2 ± 0.95^{ab}	15.0 ± 0.76^{bcd}	18.6^{b}
	Spray interval (mean)	19.9^{a} ±0.77	15.4^{b} ±0.63	
Late Season	*Annona squamosa*	16.7 ± 0.80^{bc}	11.0 ± 0.63^{bc}	13.9^{bc}
	Cashew nut	15.3 ± 0.96^{bc}	8.60 ± 0.59^{c}	12.0^{bc}
	Control	8.33 ± 0.57^{c}	8.33 ± 0.57^{c}	8.33^{c}
	Moringa oleifera	18.8 ± 0.56^{ab}	12.0 ± 0.44^{bc}	15.4^{b}
	Lambda-cyhalothrin	26.7 ± 0.57^{a}	26.7 ± 0.57^{a}	26.7^{a}
	Tephrosia vogelii	18.03 ±1.08^{ab}	11.8 ± 0.58^{bc}	14.9^{b}
	Spray interval (mean)	17.3^{a} ±0.76	13.1^{b} ±0.56	

Means with the same superscript(s) along the column are not significantly different at 5 % probability using DMRT.

plants produced the highest yield (22.2 t ha^{-1}) followed by *M. olefera* treated plots in the early planting season at 7-day intervals during early planting season. There was no significant difference ($p < 0.05$) between the plots sprayed with *T. vogelii* and *M. oleifera* at 10-day intervals in respect to yield.

However, significant higher yield was obtained from the plots sprayed with *T. vogelii* and *M. oleifera* at 7-day intervals during late planting season but yield obtained from *A. squamosa* treated plots was significantly the same as the yield obtained from *M. oleifera* and *T. vogelii* treated plots.

4 DISCUSSION

The level of insect infestation determines the efficacy of the applied insecticides. This experiment revealed that the protected plants had lower insect infestation than unprotected plants. This is an indication that plants sprayed with *T. vogelii, M. oleifera, A. squamosa* and *C. occidentale* extracts had significant insecticidal action in the control of *Monolepta* spp*., A. africana* and *C. occidentalis*. However, the insecticidal potential exhibited by these plant extracts can be attributed to the insecticidal active compounds isolated from each tested plant extracts. Rotenone has been reported by Neuwinger (2004), Sileshetal. (2005), Nyirendaet al. (2010) as insecticidal compound isolated from *T. vogelii*. Rotenone has also been described as a mitochondria poison, which blocks the electron transport chain and prevents energy production (Hollingsworth et al., 1994). Research has shown that quercetin and kaempferol derived from *M. oleifera* have been reported as insecticidal compounds (Pace-Asciak et al., 1995). The efficacy of *M. oleifera* in the control of field and stored products insect pests has been reported (Alao and Adebayo, 2015; Babarinde et al., 2013; Ileke and Olotuah, 2012). Acetenogins and anacardic acid reportedly isolated from *A. squamosa* and *A. occidentalium* respectively were also found to be effective against insect pests infestations (Olotuah and Ofuya, 2010; Oparaeke and Olubunmi, 2006).

The data suggest that the effectiveness of each test plant extracts depends on the interval of spraying. Plant sprayed at 7-day interval of spraying had higher efficacy against the observed insects than that of 10-day spraying interval. This suggests that environmental factors such as wind and sunlight reduce the potency of the applied plant extracts through degradation (Eileen and Sydney, 2013) meaning that the longer the plant extracts stay on the plant the easier the reduction in the insecticidal potential. This characteristic nature of the plant extracts is the major limitation of the plant extract as an insecticide (Isman, 2006). Based on this, botanical insecticides are not true insecticide, but they form a basis for the formulation of new synthesized insecticides, which are environmentally friendly to our farmers, consumers as well as beneficiary insects such as bees.

These results suggest that plant extracts were not effective as synthetic insecticide (lambda-cyhalothrin). This can be attributed to the photostability nature of the synthetic insecticide used. This showed that consumption of the synthetic insecticide treated fruits may have negative health impact on the human health. This may likely boost the economy of our poor resource farmers, because of their availability of the tested plant extracts. However, the preparation of plant-based insecticides does not call for any technicality.

Yield has been reported as the ultimate goal of farmers, but insect pests infestations have been described as the major factor that hindered the hope of resource farmers (Alao, 2015). Yield obtained from the plots sprayed at 10-day intervals was significantly lower compared with the yield obtained from the plots sprayed at 7-day intervals. This might have been due to significant higher level of insects attacked on the plants sprayed at 10-day intervals. This also suggest that the observed insect contribute negatively to the yield of watermelon.Lambda-cyhalothrin had higher insecticidal efficacy against insect infestations, when compared with plant extracts during early planting season. This can be attributed to photostability nature of synthetic pyrethroid (Pedigo and Rice, 2014). However, the efficacy of plant extracts as insecticides was comparable with lambda-cyhalothrin during late planting season, this is due to low level of insect infestation observed during the late planting season.

5 CONCLUSION

This experiment shows that plant extracts can be used in the management of insect pests of watermelon. Meanwhile, *T. vogelii* and *M. oleifera* exhibited higher insecticidal potential than *A. squamosa* and *A. occidentalis*. The tested plant extracts had short lasting insecticidal effects when compared with lambda-cyhalothrin, which actually killed the observed insects. This delayed effect is the major characteristic of botanical insecticides Isman, 2008). Any of these plants extracts can be applied at 7-day intervals. The use of plant extracts in the management of field and stored product pests in the developing countries will be a welcome development in the pest management program.

6 REFERENCES

Alao F.O., Adebayo, T.A. (2011). Comparative toxicity of botanical and synthetic insecticides against major field insect pests of cowpea (*Vigna unguiculata*(L.) Walp.). *Journal of Natural Product and Plant Resources, 1*(3), 86-95.

Allwood A.J., Chinajariyawong A., Drew R.A.I., Hamacek E.L., Hancock D.L., Hengsawad C., Kristaneepaiboon S., Leong C.S.T., and Vijansegaran S. (1999). Host plant record of fruitflies (Diptera; Tephritidae) in southeast Asia. *The Raffles Bulletin in zoology supplies*. *7*, 92pp.

Al-Zahrani R.M. (2007). *Systematic study of the genus Tephrosia* Pers. (Fabaceae) *in Saudi Arabia*. Post graduate thesis.

FAO (2005). *FAO STAT*: Available at http:/faostat.fao.org

FAO (2006). *FAO STAT* Agriculture Data. [Internet] Http://apps.fao.org/page/collections?subset=agriculture. Accessed

Cronquist A.C. (1993). *An integrated system of classification of flowering plants*. Columbia University Press. New York, NV

Dane F., Liu J. (2007). Diversity and origin of cultivated and citron type watermelon (*Citrullus lanatus*). *Genetic Resources and Crop Evolution*, *54*, 1255-1265. https://doi.org/10.1007/s10722-006-9107-3

Eileen A.B., Sydney G.P. (2013). *Natural Products for Managing Landscaping and Garden Pests in Florida*. EDIS.

Fahey J.W. (2005). *Moringa oleifera*: A review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1. *Trees Life J*., 1:5 (http://www.ttljournal.org/images/articles/20051201124931586_3.pdf).

Gichimu B.M., Owuor B.O., Mwai G.N., Dida, M.M. (2009). Morphological characterization of some wild and cultivated watermelon (*Citrullus* sp.) accession in Kenya. *ARPN Journal of Agricultural and Biological Science, 4*, 1990-6145.

Huh, Y.C. (2008). Morphological characterization of Korean and Turkish watermelon germplasm. Cucurbitaceae. *Proceedings of the 9th. EUCARPIA meeting on genetics and breeding of Cucurbitaceae (Pitrat M. Ed.)*, INRA, Avignon, France. May 21- 24.

Hollingworth R.M., Ahmmadsahib K.L., Gadelhak G., McLaughlin J.L. (1994). Inhibitors of complex I in the mitochondrial electron transport chain with activities as pesticides. *Biochemical Society Transactions*, *22*, 230-233. https://doi.org/10.1042/bst0220230

Ibrahim B., M'batchi B., Mounzeo H., Bourobou HP., Passo P. (2000). Effect of *Tephrosia vogelii* and *Justicia extensa* on tilapia nilotica in vivo. *Journal of Ethnopharmacology*, *69,* 99-104. https://doi.org/10.1016/S0378-8741(99)00036-7

Isman, M.B. (2006). Botanical insecticides, deterrents and repellents in modern agriculture and increasingly regulated world. *Annual Review of Entomology*, *51*, 45-66. https://doi.org/10.1146/annurev.ento.51.110104.151146

Leatemia J.A., Isman M.B. (2004). Insecticidal activity of crude seed extracts of *Annona* spp., *Lansium domesticum* and *Sandoricum koetjape* against lepidopteran larvae. *Phytoparasitica*, *32*(1), 30-37. https://doi.org/10.1007/BF02980856

Mukhopadhyay A.K., Hati A.K., Tamizharasu W., Satyababu P. (2010). Larvicidal properties of cashew nut shell liquid *(Anarcardium occidentale* L.) on immature stages of two mosquito species. *Journal of Vector Borne Diseases*, *47*, 257-260.

Neuwinger H. D. (2004). Plants used for poison fishing in tropical Africa. *Toxicon*, *44*, 417-430. https://doi.org/10.1016/j.toxicon.2004.05.014

Nyirenda S.P., Sileshi G., Belmain S.R., Kamanula J.F., Mvumi B., Sola P., Nyerenda G.K.C., Stevenson P.C. (2011). Farmers ethno-ecological knowledge of vegetable pests and their management using pesticidal plants in northern Malawi and Eastern Zambia. *African Journal of Agricultural Research*, *6*, 1525-1537.,

Opareake A.M., Bunmi B. (2006). Insecticidal potential of cashew (*Anacardium occidentale* L.) for control of the beetle, *Callosobruchus subinnotatus* (Pic, 1914) (Bruchidae) on bambara groundnut *(Voandzeia subterranea* L.*). Archives of Phytopathology and Plant Protection, 39*(4), 247 – 251. https://doi.org/10.1080/03235400500094431

Pace-Asciak C.R., Hahn S., Diamandis E.P., Soleas G., Goldberg D.M. (1995). The red wine phenolics transresveratrol and quercetin block human platelet aggregation in eicosanoid synthesis: Implication for protection against coronary heart disease. *Clinica Chimica Acta-Journal*, *235*(2), 207-219. https://doi.org/10.1016/0009-8981(95)06045-1

Qureshi R., Bhatti G.R., Memon R.A., (2010). Ethnomedicinal uses of herbs from northern part of Nara desert, Pakistan. *Pakistan Journal of Botany*, *42*, 839-851.

Schippers, R.R. (2000). African indigenous vegetable, pp: 56-60. *An overview of the cultivated species chatthan*, U.K RN. RACO, EU.

Silesh G., Mafongoya P.L., Kwesiga F., Nkunika P. (2005). Termite damage to maize grown in agro forestry systems; traditional fallows and monoculture on nitrogen-limited soils in eastern Zambia. Agriculture Forest Entomology, 7, 61-69. https://doi.org/10.1111/j.1461-9555.2005.00242.x

Oltuah O.F., Ofuya T.I. (2010). Comparative protection of cowpea, Vigna unguiculata, (L.) Walpers against field insect pests using Cashew nut shell liquid and Cypermethrin (Cymbush). Pakistan *Journal of Nutrition*, 9(2), 158161. https://doi.org/10.3923/pjn.2010.158.161

Varghese T., Pundir Y. (1964). Anatomy of the pseudocarp in *Anacardium occidentale* L. *Proceedings of the Indian Academy of Plant Sciences-Section B*, 59, 252-258.

Shukla A. (2011). The magic remedy of Oleifera: overview. *International Journal of Biomedical and Advance Research, 2*(05). https://doi.org/10.7439/ijbar.v2i6.35

doi:10.14720/aas.2020.116.1.1035

Identification of responsive genes and analysis of genes with bacterial-inducible cis-regulatory elements in the promoter regions in *Oryza sativa* L.

Abbas SAIDI [1, 2], Zohreh HAJIBARAT [1], Zahra HAJIBARAT [1]

Received January 28, 2019; accepted July 06, 2020.
Delo je prispelo 28. januarja 2019, sprejeto 06. julija 2020.

Identification of responsive genes and analysis of genes with bacterial-inducible cis-regulatory elements in the promoter regions in *Oryza sativa* L.

Abstract: Bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is one of the most critical diseases in rice. In order to study rice responsive genes to bacterial stress, microarray data were retrieved from GEO dataset. To identify the responsive genes to biotic stress (bacteria) bioinformatic tools were employed and the data presented in the forms of heatmap, gene ontology, gene network, and cis-element prediction were used. Almost all responsive genes were down-regulated at around 3 h time point and up-regulated 24 h time point in response to bacterial stress in rice varieties (*Oryza sativa* subs. *japonica* 'IR64', 'IRBB5', 'IRBB7' and 'Y73'). Gene ontology showed that genes are involved in different biological processes including translation and cellular protein metabolic processes. Network analysis showed that genes expressed in response to pathogen infection (*Xoo*) included protein translation, eukaryotic initiation factors (eIFs), ribosomal proteins, protein ubiquitin, and MAPK genes. The genes expressed in response to bacterial stress can enable plant balance between synthesis and degradation of proteins which in turn allows plants for further growth and development. TATA-box and CAAT box had the highest number of cis elements involved in bacterial stress. These genes can provide novel insights into regulatory mechanisms in biotic stress responses in rice. Identification of bacterial stress response/tolerance genes of rice can assist the molecular breeding of new rice varieties tolerant to bacterial stress.

Key words: rice; differentially-expressed genes (DEGs); bacterial stress; gene ontology; gene network

Določanje odzivnih genov in analiza genov, ki jih v promotorskih območjih riža (*Oryza sativa* L.) inducirajo bakterijski cis-regulatorni elementi

Izvleček: Bakterijski ožig riža, ki ga povzroča vrsta *Xanthomonas oryzae* pv. *oryzae* (Xoo) je ena izmed najbolj kritičnih bolezni riža. Za preučevanje odzivnih genov pri rižu na bakterijski stres so bili pridobljeni podatkovni seti eksperimenta mikromrež iz podatkovne baze GEO. Za določanje odzivnih genov na bakterijski stres so bila uporabljena bioinformacijska orodja, katerih rezultati so bili predstavljeni v obliki barvnih vizualizacij, genske ontologije, genskega omrežja in napovedi cis-elementov. Skoraj pri večini odzivnih genov je bilo izražanje zavrto po približno 3 urah in aktivirano po približno 24 urah kot odziv na bakterijski stress pri sortah riža kot so *Oryza sativa* subspecies *japonica* 'IR64', 'IRBB5', 'IRBB7' in 'Y73'. Genska ontologija je pokazala, da so ti geni vključeni v različne biološke procese, vključno s translacijo in presnovnimi procesi proteinov v celici. Analiza genskega omrežja je pokazala, da so geni, ki so se izrazili kot odziv na okužbo s patogenom (*Xoo*) vključevali translacijo proteinov, eukariontske iniciacijske faktorje (eIFs), ribosomalne proteine, protein ubikvitinin in MAPK gene. Geni, ki se izrazijo kot odziv na bakterijski stres omogočajo rastlini usklajevanje med sitezo in razgradnjo beljakovin, kar ji omogoča nadaljno rast in razvoj. Zaporedja nukleotidov kot so TATA in CAAT območja so imela največje število cis elementov povezanih z bakterijskim stresom. Ti geni lahko dajo nove vpoglede v regulatorne mehanizme pri odzivu riža na biotski stres. Identifikacija na bakterijski stres tolerantnih in odzivnih genov lahko pripomore pri molekularnem žlatnjenju novih sort riža, tolerantnih na bakterijski stres.

Ključne besede: riž; diferencialno izražanje genov (DEGs); bakterijski stres; genska ontologija; genske mreže

---

[1] Shahid Beheshti University, Faculty of Life Sciences and Biotechnology, Department of Plant Sciences and Biotechnology, Tehran, Iran
[2] Corresponding author, e-mail: abbas.saidi@gmail.com

1 INTRODUCTION

Rice (*Oryza sativa* L.), the world's most important staple crop, is the primary source of food for more than three billion people (Dawe, 2000). Rice production is affected by both biotic and abiotic stresses (Sharma et al., 2012). Bacterial blight is one of the most destructive diseases of rice causing serious yield loss to rice varieties (Sharma et al., 2012; Agrawal et al., 2002). Rice has been used as a model crop in host-pathogen interactions, bacterial pathogenesis and defense responses in monocotyledonous plants (Li et al., 2006). Recently, high-throughput techniques have provided appropriate opportunities for detecting complex signaling pathways and genetic networks involved in response to biotic and abiotic stresses (Meena et al., 2017). One of the most important techniques is microarray analysis that can be employed for such analysis. Microarray analysis showed that hundreds of genes involved in MAP cascade, hormone signaling, cell wall, and primary and secondary metabolism were induced or inhibited after Xoo infection (Grewal et al., 2012). According to other studies, genes induced and expressed in response to abiotic and biotic stresses include eukaryote initiation factors (eIF3), the DEAD-box (eIF4), ribosomal proteins (RPL and RPS), MAPK kinases, peptidases, and ubiquitin genes. Ribosomal protein (RP) is a critical factor in joining the 40S and 60S ribosomal subunits into a functional 80S ribosome (Eisinger et al., 1997). RPs have essential roles in stabilizing the ribosomal complex and mediating polypeptide synthesis in addition to extra-ribosomal functions such as their involvement in response to the environmental stresses (Sormani et al., 2011). Plants can balance between protein synthesis and degradation which in turn will allow them to grow further and develop.

In the present study, an effort has been made to dissect the rice-bacterial interaction system at 3, 6, 12, and 24 hours after inoculation (hai) in IR24, IRBB5, IRBB7, Y73 hosts based on publicly available data sets (Chen et al., 2016). Infection with *Xanthomonas oryzae* pv. o*ryzae*, leads to up-regulation of a large number of RPS genes by BLB. It has been reported that RP genes were significantly up-regulated in response to abiotic and biotic stresses in rice (Moin et al., 2016; Sormani et al., 2011). For resistance, controlling the turnover of a protein is a fundamental regulatory mechanism in post-translational modifications in plants, allowing rapid response and improved plant adaptation to internal and external signaling produced by changing environmental conditions (Boycheva et al., 2015). The aims of this work included identification of genes responsive to bacterial stress, genes associated with translation, ribosomal proteins, protein biosynthesis, and signaling pathways. Further, cis-regulatory elements in the promoter regions of the up-regulated genes were predicted.

2 MATERIALS AND METHODS

Pathogen resistance microarray data were downloaded from NCBI GEO database (www.ncbi.nlm.nih.gov/geo/) in response to bacterial pathogen Xanthomonas oryzae pv. oryzae in *Oryza sativa* (subspecies *japonica* 'IR64', 'IRBB5', 'IRBB7') represented by GSE33411 data set and ('Y73') variety represented by GSE36093 dataset (Chen et al., 2016). These datasets consist of transcriptome data with three biological replicates (a total of 51 samples). To study the regulation of genes at time points, the genes were analyzed from a range of 3 h to 24 h in response to bacterial infection.

Analysis of data was carried out by fold change which was based on log2 ratio, followed by normalizing the data using Robust Multiarray Average (RMA) method (Bolstad et al., 2003) and applied to Affymetrix data using R software with the affy package. Then the ratio expression of the genes examined under 3 h to 24 h in response to bacterial infections were compared to control and the DEGs were selected by *p*- < 0.05 as the statistical significance. The filtered data with DEGs > 2 was considered as up-regulated genes and DEGs < -2 was considered as down regulated. The probe IDs were converted into Affymetrix IDs to gene names and to detect the function of genes affected by oryzaexpress database (http://bioinf.mind.meiji.ac.jp/OryzaExpress/ID_converter). Probe sets were mapped to the MSU Rice Genome Annotation Project gene set (release 6.1). In this study, analysis of data was performed by fold change which was based on log2 ratio, followed by normalizing the data (the ratio expression of the genes examined under bacterial infection to control). BAR database (http://bar.utoronto.ca/welcome.htm) was used to identify co-expressed genes and to draw the protein-protein interaction networks (Szklarczyk et al., 2016). Single probes were matched with only one ID gene. In the datasets, samples and genes were filtered to reduce missing data between -2 < to < 2 fold changes. Gene ontology analysis was carried out using the Singular Enrichment Analysis (SEA) tool offered by agriGO (Du et al., 2010).

2.1 THE NETWORK OF CO-EXPRESSED GENES

The Pearson correlation coefficient of bacterial rice genes were obtained from the BAR database. The interactions among all genes were performed using the "The Rice Interactions Viewer" web in BAR (http://bar.utoronto.ca/welcome.htm) (Hossain et al., 2016). Nodes are colored according to its subcellular localization, edges are colored according to its co-expression correlation, and the line thickness correlates to CV. Queries produced a database of 37472 predicted and 430 confirmed Rice interacting proteins (http://bar.utoronto.ca/welcome.htm).

2.2 EXPRESSION STUDY OF BACTERIAL GENES

In order to figure out the differential gene expression (DEGs) under developmental stage, bacterial gene expression data was extracted by genevestigator from *O. sativa* database using Affymatrix Rice Genome Array platform (OS_AFFY_RICE-0) and 'Perturbations' tool. DEGs with $p < 0.05$ and log fold-change values ≥ 2 and ≤ -2 were selected for genes. Genevestigator (https://genevestigator.com/gv/) was utilized to determine the expression of bacterial genes in different developmental stages in rice.

2.3 PREDICTION OF CIS REGULATORY ELEMENTS

To access the presence of bacterial stress-responsive cis-regulatory elements, the nucleotide sequences of_1 kb upstream of each gene was retrieved from the database and submitted to the Plant Cis-Acting Regulatory Elements database (PlantCARE) (http://bioinformatics.psb. ugent.be/webtools/ plantcare/html/) (Lescot et al., 2002). PlantCARE program is designed to identify new regulatory elements under *in silico* from transcriptome data. The database can be queried on names of transcription factor (TF) sites, motif sequence, function, species, cell type, gene, TF and literature references.

3 RESULTS AND DISCUSSION

In this study, we surveyed responsive genes to bacterial stress using different bioinformatics databases. Here, we have described names and alternate names of genes, upstream promoter of significant genes, gene ontology, and expression of their genes (Table 2). Genes were down-regulated at around 3 h time point and were up-regulated at 24 h time point in response to bacterial stress in all rice cultivars (IRRB5, IRRB7, IR64, and Y73). The down-regulated genes at around 3 h time point can be assumed to have a pivotal role in early defense signaling and might have a function in reconstructing the proteins synthesis apparatus (Ouyang et al., 2007). Thus, the genes up-regulated after 24 hours of stress may interfere with the synthesis and degradation of proteins.

3.1 DETECTION OF RESPONSIVE-GENES INVOLVED IN CO-EXPRESSED GENE NETWORK

The network analysis of significant genes (in total 251) is given in Figure 3. Nodes are illustrated according to protein subcellular localization, edges are illustrated according to its co-expression correlation and the line thickness correlates to CV. Alternatively, based on rice gene expression data sets available in the BAR database, nodes can be colored according to their expression levels in specific organelles (Toufighi et al., 2005). For each edge connecting a pair of proteins, the RIV is able to show different colors based on the co-expression value (PCC). Overall, for a pair of proteins to physically interact, each protein must be located at the same or adjacent subcellular compartments either permanently or transiently.

In the network, LOC_Os01g66520 seemed to be the central protein encoding the "Serine/Threonine Protein Kinase" (STK). Most of the proteins in this network seem to be localized mainly in nucleus (blue), cytoplasm (pink), chloroplast (green), and mitochondria (light blue). These proteins encode mainly for the significant genes such as LOC_Os04g30780 (eIF-3h), LOC_Os09g36300 (OsLon4), LOC_Os05g01450 (eIF-3f), LOC_Os02g55370 (RPL39), and LOC_Os03g08010 (EF-TU) (Figure 1). Genes involved in the network analysis such as Serine/Threonine Protein Kinase (STK) pathways and translation protein genes (eIFs, ribosomal protein, EF-Tu) have been found to play important roles in response to *Xanthomonas oryzae* (Grewal et al., 2012). Serine/Threonine Protein Kinase (STK) is involved in the disease resistance signaling process (Ülker & Somssich 2004). Accroding to other studies, the transcriptional activity of MPKs has been observed in response to several biotic stresses (Agrawal et al., 2002Complexity of the genetic networks involved in defense response has also been reported in MPKs. Based on previous results, several stress-responsive genes (MPKs) are regulated by biotic and abiotic stresses (Ghanashyam & Jain, 2009; Jain & Khurana, 2009).

Proteins expressed in the nucleus include LOC_Os01g03070 (eIF-3a (transposon), LOC_Os05g01450 (eIF3-F), and LOC_Os03g59310 (ribosomal protein). Analysis of genes network showed up-regulation of ribosomal proteins, *eIFs* genes, and MAPK genes. Kolupaeva and colleagues suggested that eIF3 plays an important role in separating the subunits in the terminal

Table 1: Characteristics of responsive genes to bacterial stress

No	Affymetrix probe ID	MSU7	RAP-ID	Function
1	Os.10120.1.S1_at	LOC_Os06g36160	Os06g0555400	40S ribosomal protein S19-like
2	Os.10132.1.S1_at	LOC_Os09g36300	Os09g0533400	OsLon4 (Putative Lon protease homologue)
3	Os.10955.1.S1_at	LOC_Os02g54700	Os02g0788400	eIF-3g
4	Os.11049.1.S1_at	LOC_Os03g59310	Os03g0807800	Ribosomal protein
5	Os.11168.1.S1_x_at	LOC_Os02g55370	Os02g0796900	60S ribosomal protein L39 (RPL39)
6	Os.11420.1.S1_at	LOC_Os05g01450	Os05g0104800	eIF-3f
7	Os.12189.2.S1_x_at	LOC_Os11g38959	Os11g0602600	40S ribosomal protein S9
8	Os.12413.1.S1_at	LOC_Os03g22340	Os03g0343500	60S ribosomal protein L22-2 (RPL22-2)
9	Os.12625.1.S1_a_at	LOC_Os03g08010	Os03g0177400	Elongation factor Tu (EF-Tu)
10	Os.12772.1.S1_at	LOC_Os03g13170	Os03g0234200	Ubiquitin fusion protein
11	Os.139.1.S1_at	LOC_Os06g48750	Os06g0701100	eIF-4a (DEAD-box ATP-dependent RNA helicase)
12	Os.14437.1.S1_at	LOC_Os01g66520	Os01g0888700	Serine/threonine-protein kinase (STK)
13	Os.14589.1.S1_at	LOC_Os03g56241	Os03g0773150	40S ribosomal protein S29
14	Os.28076.1.S2_at	LOC_Os01g03070	Os01g0120800	eIF-3a (transposon)
15	Os.38164.1.S1_at	LOC_Os12g38180	Os12g0569700	OsHsp23.7
16	Os.40424.1.S1_at	LOC_Os01g54340	Os01g0747300	Plant-specific domain TIGR01615 family protein
17	Os.41468.1.S1_at	LOC_Os01g47050	Os01g0659900	OsFbox033
18	Os.4714.1.S1_at	LOC_Os07g01870	Os07g0109500	Ribosomal protein L13 family protein (RPL13)
19	Os.47337.1.S1_at	LOC_Os02g10820	Os02g0202600	Sel1 repeat domain containing protein
20	Os.5326.1.S1_a_at	LOC_Os05g02880	Os05g0120100	Splicing factor
21	Os.7573.1.S1_at	LOC_Os06g49140	Os06g0704900	Ribosomal RNA large subunit methyltransferase J
22	Os.8580.1.S1_at	LOC_Os04g30780	Os04g0376500	eIF-3h (Eukaryotic translation initiation factor 3 subunit H)
23	OsAffx.4176.1.S1_at	LOC_Os04g54640	Os04g0639300	Peptidase

codon (Kolupaeva et al., 2005). Further, eIF3 gene has been shown to improve stress tolerance and thus can be utilized through transgenic approaches. Similar studies confirming our results suggest that large and small subunits of ribosomal protein provide a better understanding of stress response in rice (Hummel et al., 2012; Saidi & Hajibarat, 2019).

Cytosolic localized proteins are estimated to interact with nuclear and mitochondrial localized proteins, providing possible import/ export regulation proteins such as transcription factors (Ho et al., 2012). In addition, several proteins are also expressed in the vacuole including LOC_Os03g05980 (protein 40S ribosomal protein S9-2) and LOC_Os01g03070 (protein translation initiation factor SUI1, putative, expressed).

Analysis of network showed that only a few proteins are located in each of the chloroplast, nucleus, cytoplasm and vacuole. LOC_Os07g01870 (RPL13) and LOC_Os07g02340 (RPL22-2) are mainly translational proteins localized in the chloroplast (Figure 1). For resistance to the bacterial stress, large and small subunits of ribosomal genes (RPS and RPL), associated with proteins translation, are regulated. Other genes associated with translation of proteins include up-regulation of initiation and elongation factors. In addition, peptidase, ubiquitin, and OsLonP4 protease genes are expressed in response to bacterial stress. Further, serine / threonine kinase genes are also expressed in response to bacterial stress.

3.2 DIFFERENTIALLY-EXPRESSED GENES IN RESPONSE TO DEVELOPMENTAL STAGES

Microarray data indicated an up regulation of significant genes during different developmental stages; this may be due to the presence of different cis elements (Figure 4). As a result, these genes can play key roles in different growth and developmental stages under abiotic and biotic stresses. RPS and RPL genes were also up-regulated during different developmental stages indicating that both can play important roles in multiple biological processes, protein synthesis, cell growth, development, and apoptosis (Warner & McIntosh, 2009). Ribosomal

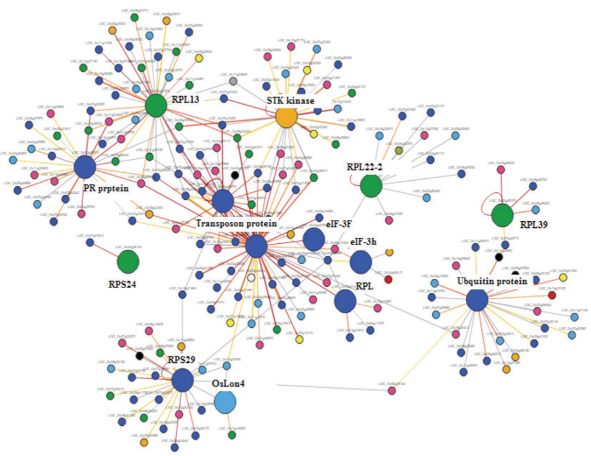


Figure 1: Predicted gene network for responsive genes to bacterial stress, drawn up by the BAR database where the drawn lines represent the relationships among genes.

proteins (RPs) are vital in ribosome biogenesis and protein synthesis and play a key role in diverse developmental processes (Saha et al., 2017).

3.3 ANALYSIS OF RPL AND RPS IN RESPONSE TO BACTERIAL STRESSES

RPs have long been known primarily for their housekeeping functions. RPL13, RPS7, and RPS9-2 responded to bacterial stresses and biotic stress. However, there are several reports showing RPs having several functions under different stress conditions (Warner &McIntosh, 2009). Out of the 80 RPs encoded by 3200 genes in *Arabidopsis*, 32 are small subunits and 48 are large subunits (Wang et al., 2013). In rice, there are 34 large protein subunits with multiple gene copies encoding ribosomal proteins (McIntosh & Bonham-Smith, 2006: Moin et al.2016).

Although many of the RPS and RPL genes are expressed in response to bacterial stress, however, they cannot be considered as house-keeping as their level of expression changes in response to environmental signals (Moin et al., 2016). Based on microarray data, we have suggested that RPL and RPS genes might be useful as candidate genes for manipulating stress tolerant rice varieties.

Very few studies have been conducted on RPL and RPS genes of rice (Moin et al., 2016; Wang et al.2003). We performed an extensive analysis on the expression of all ribosomal genes (RPS) induced in rice. Differential expression of RPS and RPL genes under bacterial stress condition significantly increased expression. RPL and RPS genes can be induced under different stresses such as heat, cold, low irrigation, and pathogens (Wang et al., 2003). Each stress activates a series of cascade signaling pathways for plant survival and plant growth. Up-regulation of ribosomal genes and the presence of several regulatory elements in the upstream regions of the genes promoter (Table 1) confirm other findings that these genes may play an important role in reducing the biotic and abiotic stresses in rice (Fujimoto et al., 2000).

It has been reported that over-expression of RPL13 enhances resistance against a fungal pathogen (*Verticillium dahliae* Kleb.*)* in transgenic potato as well as up-regulation of genes coding for defense and antioxidant enzymes (Yang et al., 2015; Song et al., 2020). In addition, *RPS7* and *RPS9-2* genes were up-regulated in response to bacterial stress. Our findings are in agreement with others who have reported that these genes are also up-regulated in response to PEG and salt treatment (Kawasaki et al., 2001). Based on our findings it can be suggested that *eIFs*, *RPL*, and *RPS* genes can be particularly promising in bringing about the tolerance to multiple stresses in rice.

Recent studies in different plant species have shown that the lack of *eIF4A* (DEAD-box) gene leads to decreased root formation, delayed flowering, and abnormal ovule formation suggesting that eIF4A plays an essential role in growth and development of plants (Peng et al., 2008; Maršálová et al., 2016). The *Arabidopsis* genome encodes eIF4A, required for the coordination between cell cycle progression and cell size (Bush et al., 2015; Saidi & Hajibarat, 2020).

3.4 FUNCTIONAL CLASSIFICATION OF OVERLAPPING GENES

Based on gene ontology (GO) classification, genes

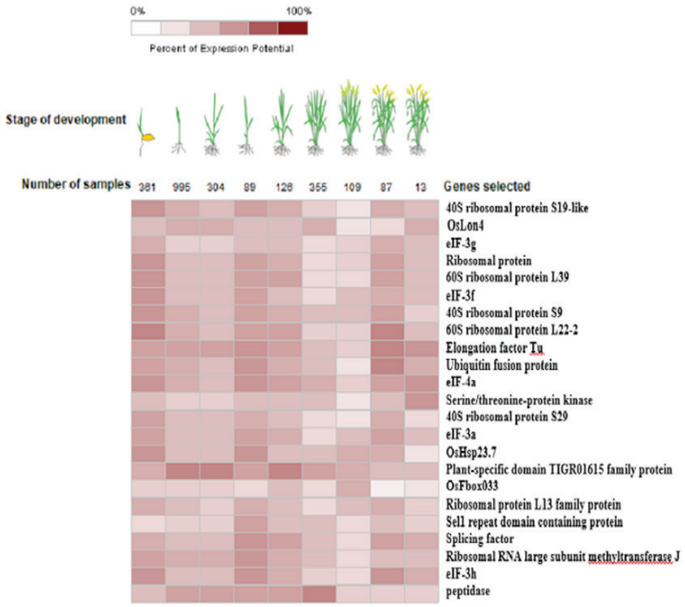


Figure 2: Heatmap representation of expression analysis of bacterial responsive genes at different developmental stages of *O. sativa*..

Table 2: Analysis of gene ontology and KEGG pathway in response to bacterial stress in rice

	pathway ID	pathway description	count in gene set	false discovery rate
	GO:0006412	Translation	4	0.000113
Biological Process (GO)	GO:0044267	cellular protein metabolic process	5	0.00139
	GO:0010467	gene expression	5	0.0029
Molecular Function	GO:0003735	structural constituent of ribosome	3	0.00361
Cellular Component	GO:0005840	Ribosome	3	0.00195
KEGG Pathways	03010	Ribosome	8	1.88e-11
	03013	RNA transport	5	3e-07

responding to different treatments were categorized into different functional groups of biological processes, cellular components and molecular functions (Table 2). In the biological process category, translation, peptide biosynthetic processes and amide biosynthetic processes were the most dominant groups. In the cellular components group, ribosome subunit was the largest group, followed by intracellular and non-membrane organelles. Structural constituent of ribosome was the largest group in the molecular function, followed by structural molecule activity. Gene ontology showed that most genes are involved in translation and protein biosyntesis. GO analysis of all genes showed that the lowest gene counts responsible for translation included four genes and the highest gene count responsible for cellular protein metabolic processes and gene expression included five genes (Table 2). According to our GO enrichment analysis, significant genes had translational function in protein biosynthesis pathway. The balance between protein synthesis and degradation is necessary to maintain and improve homeostasis and plant survival (Boycheva et al., 2015). All responsive genes were involved in both KEGG pathways including ribosome (pathway ID: 03010) and RNA transport (pathway ID: 03013) with ribosome having the highest gene count (Table 2).

3.5 CIS REGULATORY ELEMENTS ANALYSIS

Cis regulatory elements prediction analysis resulted in the identification of multiple stress-responsive elements in the promoter regions of some genes such as eIF-3g, eIF-4a, RPL39, RPL13, and eIF-3h (Figure 3). In addition to abiotic stresses, cis elements also respond to phyto-hormones such as ABA (ABRE-Abscisic acid responsive element and Motif IIb), MeJa (TGACG-motif

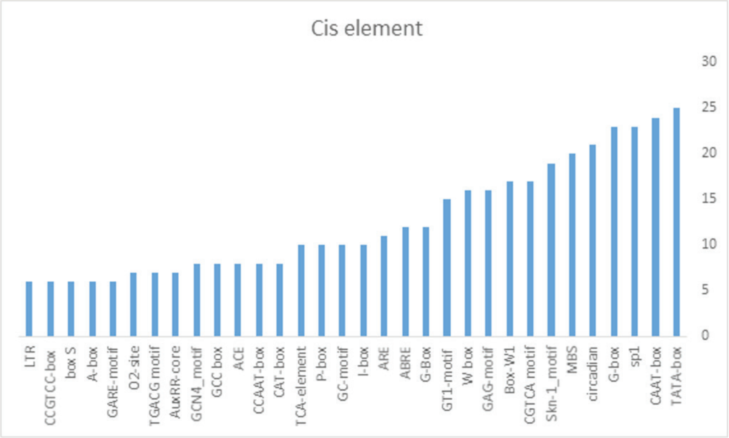


Figure 3: Cis regulatory elements detected in the region of the responsive genes and their frequency in each gene, the 1000-bp upper region of upstream of each gene were searched using the PlantCARE database (http://bioinformatics.psb. ugent.be/webtools/plantcare/html/) to identify responsive elements to bacterial stress.

and CGTCA-motif), SA (TCA-motif), Gibberellic acid (GARE- Gibberellic acid responsive element), and Auxin (TGA-motif and AuxR-Auxin responsiveness). Only few motifs such as ABRE (abscisic acid), CGTCA and TGACG (methyl-jasmonate), GCC-box (ethylene), and TCA element (salicylic acid) were present in both *A. thaliana* and *O. sativa* PRs (Kaur et al., 2017).

Our results suggest that hormones involved in bacterial stress may play a major role in regulating stress responses. Various elicitor-responsive elements such as GCC box and W box have been observed in some genes. W-box motifs are the binding sites for stress-responsive WRKY transcriptional factors (Eulgem & Somssich, 2007) and a Box-W1 motif, a fungal elicitor element that binds with WRKY33 transcriptional factor in response to phyto-pathogens (Lippok et al.2007; Saidi & Hajibarat, 2018)

A detailed analysis of both abiotic and biotic responsive elements and their repeats in the promoter regions is presented in Figure 3. Cis-regulatory elements in the promoter sequences provide a good understanding of plant responses under biotic stress conditions. Genes promoter analysis revealed that TATA-box and CAAT-box were present in majority of promoter regions of the genes. GATA-box, G-box, Box-4, GAG, GA-motif and I-box are known as light responsive cis-elements (LREs) and have been found in the regulatory region of light-regulated genes, apparently essential for light-controlled transcriptional activity (Gilmartin et al., 1990). TGACG motif is methyl jasmonate responsive element present among *Arabidopsis* and *O. sativa* PR gene sequences (Kaur et al.2017). The transcription of TGACG mediated PR sequences is regulated by binding of BZIP TGA factor to TGACG element (Wang et al., 2013). According to the studies performed on the regions of gene promoter, regulatory elements of the genes responsive to auxin and jasmonate (jasmonic acid) hormones are up-regulated in response to the infection of the pathogen, perhaps due to the presence of WRKYs in the promoter regions (Dowd et al., 2004; O'Brien et al., 2013).

4 CONCLUSION

In the present study, a comprehensive analysis of responsive genes, in terms of co-expressed network, cis-elements, gene ontology, and expression study in promoter regions in rice was performed. Analysis of promoter regions revealed the presence of different types and frequencies of cis elements among genes. In our analysis, several cis elements including TATA, CAAT-box, Sp1, and G-box were identified in the promoter regions of the responsive genes. Among the responsive genes, RPL and RPS were the most up-regulated genes during developmental stages. In this study, the identified responsive genes to bacterial stress can be utilized as candidate genes for crop breeding. Plants activate these genes to balance between the synthesis and degradation of proteins under stress conditions. This work suggests that the cis elements in the promoter regions of the genes can provide new ways for improving plant resistance against pathogens. Also, analysis of responsive gene expression revealed that these genes can be considered for rice breeding for resistance to abiotic and biotic stresses.

5 REFERENCES

Agrawal, G.K., Rakwal, R. and Iwahashi, H. (2002). Isolation of novel rice (*Oryza sativa* L.) multiple stress responsive MAP kinase gene, OsMSRMK2, whose mRNA accumulates rapidly in response to environmental cues. *Biochemical and Biophysical Research Communications*, *294*(5), 1009-1016. https://doi.org/10.1016/S0006-291X(02)00571-5

Boycheva, I., Vassileva, V., Revalska, M., Zehirov, G. and Iantcheva, A. (2015). Cyclin-like F-box protein plays a role in growth and development of the three model species *Medicago truncatula*, *Lotus japonicus*, and *Arabidopsis thaliana*. *Research and Reports in Biology*, *6*, 117-130. https://doi.org/10.2147/RRB.S84753

Bolstad, B.M., Irizarry, R.A., Åstrand, M. and Speed, T.P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*, *19*(2), 185-193. https://doi.org/10.1093/bioinformatics/19.2.185

Chen, X., Dong, Y., Yu, C., Fang, X., Deng, Z., Yan, C., & Chen, J. (2016). Analysis of the proteins secreted from the Oryza meyeriana suspension-cultured cells induced by *Xanthomonas oryzae* pv. *oryzae*. *PloS One*, *11*(5). https://doi.org/10.1371/journal.pone.0154793

Dowd, C., Wilson, I.W. and McFadden, H. (2004). Gene expression profile changes in cotton root and hypocotyl tissues in response to infection with *Fusarium oxysporum* f. sp. *vasinfectum*. *Molecular Plant-Microbe Interactions*, *17*(6), 654-667. https://doi.org/10.1094/MPMI.2004.17.6.654

Du, Z., Zhou, X., Ling, Y., Zhang, Z. and Su, Z. (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic acids research*, *38*(suppl_2), W64-W70. https://doi.org/10.1093/nar/gkq310

Eisinger, D.P., Dick, F.A. and Trumpower, B.L. (1997). Qsr1p, a 60S ribosomal subunit protein, is required for joining of 40S and 60S subunits. *Molecular and Cellular Biology*, *17*(9), 5136-5145. https://doi.org/10.1128/MCB.17.9.5136

Eulgem, T. and Somssich, I.E. (2007). Networks of WRKY transcription factors in defense signaling. *Current Opinion in Plant Biology*, *10*(4), 366-371. https://doi.org/10.1016/j.pbi.2007.04.020

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box–mediated gene expression. *The Plant Cell*, *12*(3), 393-404. https://doi.org/10.1105/tpc.12.3.393

Ghanashyam, C. and Jain, M. (2009). Role of auxin-responsive genes in biotic stress responses. *Plant Signaling & Behavior*, *4*(9), 846-848. https://doi.org/10.4161/psb.4.9.9376

Gilmartin, P.M., Sarokin, L., Memelink, J. and Chua, N.H. (1990). Molecular light switches for plant genes. *The Plant Cell*, *2*(5), 369. https://doi.org/10.2307/3869087

Grewal, R.K., Gupta, S. and Das, S. (2012). *Xanthomonas oryzae* pv *oryzae* triggers immediate transcriptomic modulations in rice. *BMC Genomics*, *13*(1), 49. https://doi.org/10.1186/1471-2164-13-49

Hummel, M., Cordewener, J.H., de Groot, J.C., Smeekens, S., America, A.H. and Hanson, J. (2012). Dynamic protein composition of *Arabidopsis thaliana* cytosolic ribosomes in response to sucrose feeding as revealed by label free MS E proteomics. *Proteomics*, *12*(7), 1024-1038. https://doi.org/10.1002/pmic.201100413

Jain, M. and Khurana, J.P. (2009). Transcript profiling reveals diverse roles of auxin-responsive genes during reproductive development and abiotic stress in rice. *The FEBS Journal*, *276*(11), 3148-3162. https://doi.org/10.1111/j.1742-4658.2009.07033.x

Kaur, A., Pati, P.K., Pati, A.M. and Nagpal, A.K. (2017). In-silico analysis of cis-acting regulatory elements of pathogenesis-related proteins of Arabidopsis thaliana and Oryza sativa. *PloS One*, *12*(9), e0184523. https://doi.org/10.1371/journal.pone.0184523

Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D. and Bohnert, H.J. (2001). Gene expression profiles during the initial phase of salt stress in rice. *The Plant Cell*, *13*(4), 889-905. https://doi.org/10.1105/tpc.13.4.889

Kolupaeva, V.G., Unbehaun, A., Lomakin, I.B., Hellen, C.U. and Pestova, T.V. (2005). Binding of eukaryotic initiation factor 3 to ribosomal 40S subunits and its role in ribosomal dissociation and anti-association. *Rna*, *11*(4), 470-486. https://doi.org/10.1261/rna.7215305

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., ... & Rombauts, S. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Research*, *30*(1), 325-327. https://doi.org/10.1093/nar/30.1.325

Li, Q., Chen, F., Sun, L., Zhang, Z., Yang, Y. and He, Z. (2006). Expression profiling of rice genes in early defense responses to blast and bacterial blight pathogens using cDNA microarray. *Physiological and Molecular Plant Pathology*, *68*(1-3), 51-60. https://doi.org/10.1016/j.pmpp.2006.06.002

Lippok, B., Birkenbihl, R.P., Rivory, G., Brümmer, J., Schmelzer, E., Logemann, E. and Somssich, I.E. (2007). Expression of AtWRKY33 encoding a pathogen-or PAMP-responsive WRKY transcription factor is regulated by a composite DNA motif containing W box elements. *Molecular Plant-Microbe Interactions*, *20*(4), 420-429. https://doi.org/10.1094/MPMI-20-4-0420

Moin, M., Bakshi, A., Saha, A., Dutta, M., Madhav, S.M. and Kirti, P.B. (2016). Rice ribosomal protein large subunit genes and their spatio-temporal and stress regulation. *Frontiers in Plant Science*, *7*, 1284. https://doi.org/10.3389/fpls.2016.01284

Meena, K.K., Sorty, A.M., Bitla, U.M., Choudhary, K., Gupta, P., Pareek, A., Singh, D.P., Prabha, R., Sahu, P.K., Gupta, V.K. and Singh, H.B. (2017). Abiotic stress responses and microbe-mediated mitigation in plants: the omics strategies. *Frontiers in Plant Science*, *8*, p.172. https://doi.org/10.3389/fpls.2017.00172

O'Brien, J.A. and Benková, E. (2013). Cytokinin cross-talking during biotic and abiotic stress responses. *Frontiers in Plant Science*, *4*, 451. https://doi.org/10.3389/fpls.2013.00451

Saidi, A., & Hajibarat, Z. (2018). In silico analysis of floral mads-box gene in *Brachypodium distachyon*. *BIONATURE*, 366-375.

Saidi, A., & Hajibarat, Z. (2019). Characterization of cis-elements in hormonal stress-responsive genes in *Oryza sativa*. https://doi.org/10.35118/apjmbb.2019.027.1.10

Saidi, A., & Hajibarat, Z. (2020). In-silico analysis of eukaryotic translation initiation factors (eIFs) in response to environmental stresses in rice (*Oryza sativa*). *Biologia*, 1-8. https://doi.org/10.2478/s11756-020-00467-1

Sharma, T.R., Rai, A.K., Gupta, S.K., Vijayan, J., Devanna, B.N. and Ray, S. (2012). Rice blast management through host-plant resistance: retrospect and prospects. *Agricultural Research*, *1*(1), 37-52. https://doi.org/10.1007/s40003-011-0003-5

Song, R., Li, J., Xie, C., Jian, W., & Yang, X. (2020). An Overview of the Molecular Genetics of Plant Resistance to the *Verticillium* Wilt Pathogen *Verticillium dahliae*. *International Journal of Molecular Sciences*, *21*(3), 1120. https://doi.org/10.3390/ijms21031120

Sormani, R., Masclaux-Daubresse, C., Daniele-Vedele, F. and Chardon, F. (2011). Transcriptional regulation of ribosome components are determined by stress according to cellular compartments in *Arabidopsis thaliana*. *PLoS One*, *6*(12), p.e28070. https://doi.org/10.1371/journal.pone.0028070

Toufighi, K., Brady, S. M., Austin, R., Ly, E., & Provart, N. J. (2005). The Botany Array Resource: e-Northerns, expression angling, and promoter analyses. *The Plant Journal*, *43*(1), 153-163. https://doi.org/10.1111/j.1365-313X.2005.02437.x

Ülker, B. and Somssich, I.E., 2004. WRKY transcription factors: from DNA binding towards biological function. *Current Opinion in Plant Biology*, *7*(5), 491-498. https://doi.org/10.1016/j.pbi.2004.07.012

Yang, L., Mu, X., Liu, C., Cai, J., Shi, K., Zhu, W., and Yang, Q. (2015). Overexpression of potato miR482e enhanced plant sensitivity to *Verticillium dahliae* infection. *Journal of Integrative Plant Biology*, *57*(12), 1078-1088. https://doi.org/10.1111/jipb.12348

Wang, W., Vinocur, B. and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, *218*(1), 1-14. https://doi.org/10.1007/s00425-003-1105-5

Wang, P., Du, Y., Zhao, X., Miao, Y. and Song, C.P. (2013). The MPK6-ERF6-ROSE7/GCC-box complex modulates oxidative gene transcription and the oxidative response in *Arabidopsis thaliana*. *Plant Physiology*, 112. https://doi.org/10.1104/pp.112.210724

doi:10.14720/aas.2020.116.1.1382 Original research article / izvirni znanstveni članek

Sweet corn crop response to different dripline spacings in the dry Mediterranean area

Ibrahim MUBARAK [1, 2] , Mussaddak JANAT [1]

Received December 16, 2019; accepted August 11, 2020.
Delo je prispelo 16. decembra 2019, sprejeto 11. avgusta 2020.

Sweet corn crop response to different dripline spacings in the dry Mediterranean area

Abstract: Increasing the spacing between driplines is the most significant factor in reducing the high initial costs of drip irrigation system. A two-year field study (2017 and 2018) was conducted on a clay loam soil to evaluate the effects of different dripline spacings (75, 150, 225, and 300 cm) on sweet corn yields, soil water status, and irrigation water use efficiency (IWUE) in the dry Mediterranean area. Results showed that the average husked cob yield (HCY) and IWUE were significantly decreased as the dripline spacing increased. HCY was 13.93, 12.30, 9.18, and 6.86 t ha^{-1}, for dripline spacing of 75, 150, 225, and 300 cm, respectively, for an average irrigation water depth of 725 mm. Both soil water storages and yields decreased with distance from the dripline. The 150-, 225- and 300 cm dripline spacings yielded 11.7, 34.0 and 50.8 % less than the 75 cm dripline spacing, but these wider dripline spacings provided 50, 67 and 75 % less unit-area cost, respectively. However, these reductions in crop yield did not justify the extra cost of the closer dripline spacing, at low crop prices and high dripline costs. Finally, further studies are needed to benefit from the higher initial unit-area cost savings obtained under the wider dripline spacings.

Key words: drip irrigation; clay loam soil; soil water storage; husked cob yield; row-to-row variations

Odziv sladke koruze na različne namestitve namakalnih naprav v suhem sredozemskem območju

Izvleček: Povečevanje razdalje med namakalnimi cevmi je najpomembnejši dejavnik pri zmanjševanju začetnih stroškov pri namestitvi namakalnega sistema. Za ovrednotenje učinkov različne namestitve namakalnih cevi (75, 150, 225, in 300 cm) na pridelek sladke koruze, stanja vode v tleh in na učinkovitost zalivanja (IWUE) je bil izveden dvoletni poljski poskus (2017 in 2018) v sušnem sredozemskem območju. Rezultati so pokazali, da sta se poprečni pridelek oličkanih storžev (ICY) in učinkovitost zalivanja (IWUE) značilno zmanjšala s povečevanjem razdalje med zalivalnimi cevmi. HCY je bil 13,93, 12,30, 9,18, in 6,86 t ha^{-1}, pri razmaku zalivalnih cevi 75, 150, 225, in 300 cm, pri poprečni globini zalivanja 725 mm. Z oddaljenostjo od namakalnih cevi sta se zmanjševala pridelek in vsebnost vode v tleh. Namestitev namakalnih cevi na 150, 225 in 300 cm je dala za 11,7, 34,0 in 50,8 % manj pridelka v primerjavi z namestitvijo na 75 cm, a je imela ta namestitev namakalnih cevi za 50, 67 in 75 % manj stroškov na enoto površine. Kljub temu zmanjšanje pridelka ni upravičilo dodatnih stroškov za gostejšo namestitev namakalnih cevi zaradi nizkih cen pridelka in visokih stroškov namakalnih cevi. Zaključimo lahko, da so potrebne dodatne raziskave prihrankov visokih začetnih stroškov na enoto površine pri večjih razmakih namakalnih cevi.

Ključne besede: kapljični namakalni sistem; ilovnato-peščena tla; zadrževanje vode v tleh; pridelek oličkanih storžev; razlike med razdaljami med namakalnimi cevmi

---

[1] Atomic Energy Commission of Syria, Department of Agriculture, Damascus, Syria
[2] Corresponding author, e-mail: ascientific10@aec.org.sy

1 INTRODUCTION

Sweet corn (*Zea mays* L. convar. *saccharata* Koern. var. *rugosa*) is one of the most popular vegetables grown for human consumption as either a fresh or processed product. Fresh consumption of sweet corn by boiling or grilling is rapidly increasing worldwide. Husked cobs of large sizes are more attractive in market. Moreover, stalks and leaves are used for animal feed. Efficient water use, where water resources are scarce as in the dry Mediterranean area, is an urgent need to address the sustainability of crop production for increasing food demands.

Drip irrigation system has been proved to improve water use efficiency and offer yield enhancement compared with sprinkle and surface irrigation (Clemmens, 2002; Tiercelin, 2007). However, one of the greatest barriers to the widespread adoption of drip irrigation is its high initial investment cost compared with the other irrigation systems. The cost of driplines (laterals) represents a large part of the initial cost. Bozkurt et al. (2006) reported that the cost of pipes (all tubing and laterals) was about 45 % of the total cost of a drip irrigation system. Therefore, adoptation of economically-sound and scientifically-proven techniques is a practical tool for reducing the high initial cost, which compiles with best management practices.

In this context, increasing the spacing between driplines would be one of the most significant factors. For example, using a dripline for two crop rows rather than for one would half the number of driplines as per crop rows, and therefore, would reduce the initial cost of irrigation system (Henggeler, 1995; Camp et al., 1997; Camp, 1998; Lamm and Trooien, 2003; Bozkurt et al., 2006; Zhou et al., 2017; Al-hurmuzi and Topak, 2018; Mubarak, 2020a and b). In regions such as the humid Mediterranean region and the wetter region of Virginia and Kansas in USA, that normally receive substantial volume of rainfall at planting time, changing dripline spacings have not greatly influenced germination or final yield; and thus yield was not sufficiently reduced to warrant the extra cost of a closer dripline spacing (Camp et al., 1989; Spurgeon and Manges, 1990; Powell and Wright, 1993; Camp et al., 1997; Lamm et al., 1997; Al-hurmuzi and Topak, 2018). On the other hand, in regions where rainfall is unreliable throughout the year, and/or near-surface soil water that is required for seed germination often evaporates due to the high climatic demand, wider dripline spacings require excessive pre-planting irrigation or using another irrigation system as sprinkle system in order

Table 1: Some climatic data for the experimental station during both growing seasons (2017 and 2018), and the average of last 20 years

Variable	Year	Apr.	May	Jun.	Jul.
	2017	9.7	14.4	17.2	20.6
	2018	10.0	15.6	18.2	19.8
T_{min} (^{0}C)	20-year average	10.1	14.1	17.6	19.3
	2017	26.2	31.6	35.7	40.6
	2018	27.2	31.5	34.6	36.9
T_{max} (^{0}C)	20-year average	25.3	30.4	35.0	37.4
	2017	19.2	24.9	28.4	31.1
	2018	19.9	25.7	27.7	28.8
$T_{average}$ (^{0}C)	20-year average	18.1	23.6	27.7	29.4
	2017	63.1	57.9	56.3	56.0
	2018	54.8	51.5	59.6	55.6
RH (%)	20-year average	60.9	56.5	56.3	60.7
	2017	5.7	7.6	9.0	10.3
	2018	6.8	7.8	8.8	10.3
ET_0 (mm day^{-1})	20-year average	5.6	7.5	9.4	10.4
	2017	0.0	0.0	0.0	0.0
	2018	14	0.0	0.0	0.0
Rainfall (mm)	20-year average	5.9	4.2	0.0	0.0

T_{min}: minimum temperature, T_{max}: maximum temperature, $T_{average}$: average temperature, *RH*: relative air humidity, ET_0: reference evapotranspiration.

to suitably germinate the seeds (Henggeler, 1995; Howell et al., 1997; Bordovsky and Porter, 2003). Failing to do so would potentially result in poor seed germination and crop establishment (Charlesworth et al., 1998; Charlesworth and Muirhead, 2003). The problem could be worse for coarser soils where water movement is dominated by gravity rather than capillary forces (Thorburn et al., 2003), or for soils that have a relatively high clay content where shrinking-swelling cycles result in large cracks (Howell et al., 1997). Moreover, even in locations where rainfall is reasonably reliable, row-to-row yield variation may increase with increasing dripline spacings. Yields from plant rows away from driplines were found to be significantly lower than those from rows adjacent to the driplines (Kruse and Israeli, 1987; Lamm et al., 1997; Bosch et al., 1998; Bozkurt et al., 2006; Mubarak, 2020a). Nevertheless, to the best of our knowledge, very few findings are presently available on the use of one dripline for three or four rows of sweet corn grown in the dry Mediterranean area. Under such arrangement, the number per unit area of driplines and, therefore, their initial cost decreased to a third or to a quarter, as compared with the conventional pattern of one dripline for each plant row.

The specific objective was to evaluate the effects of dripline spacings on sweet corn yields, soil water status, and irrigation water use efficiency. Results may introduce practical alternatives of drip-irrigated sweet corn production system to meet sustainable production and water shortage in the dry Mediterranean area.

2 MATERIALS AND METHODS

2.1 STUDY AREA

Field experiments were conducted at the Deir Al-Hajar Agricultural Experiment Station, south of Damascus, Syria (33°20′ N, 36°26′ E, altitude 600 m), for two consecutive growing seasons 2017 and 2018. The area is characterized by a dry Mediterranean type climate, with an average annual precipitation of about 120 mm (October-May). Some climate data during the course of experi-

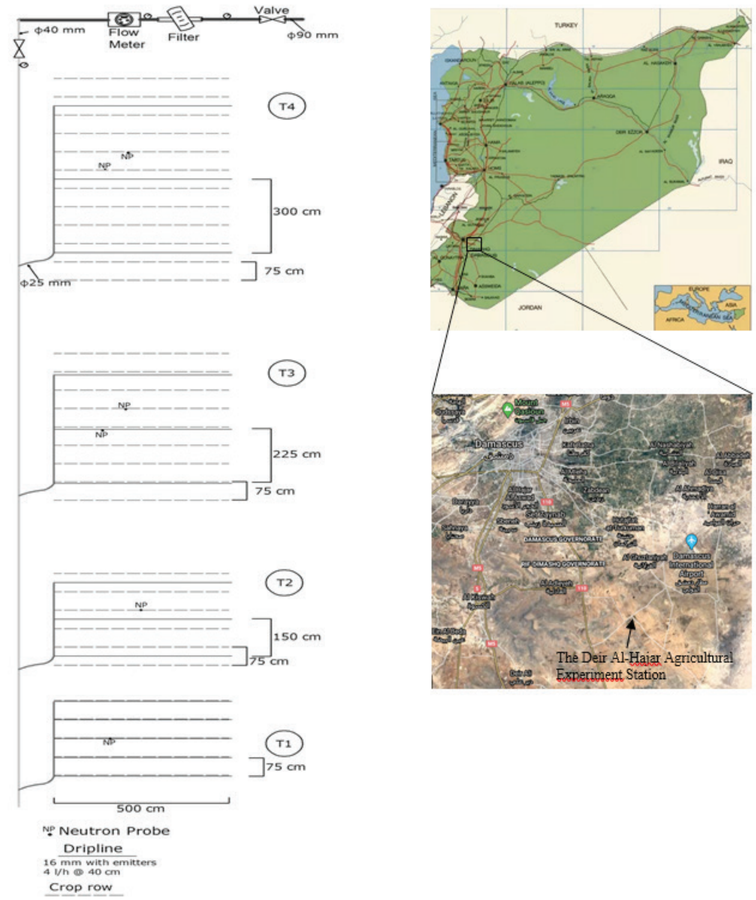


Figure 1: Outline of one replicate according to dripline spacings. T1, T2, T3, and T4 represent S75, S150, S225, and S300, respectively, and maps of the geographical position in Syria of the experimental station.

ments were collected from a weather station located close to the study site. The climate data for both years as well as the 20-year average are reported in Table 1.

The soil is classified as a clay loam with 27.8 % sand, 42.7 % silt, and 29.5 % clay, with a bulk density of about 1.35 g cm^{-3}. Before planting, the topsoil (0-30 cm) was characterized by about 1 % of organic matter, pH of 8.0, EC of 0.6 ds m^{-1}, available P of 22.0 ppm, NO_3^- 21.1 ppm, NH_4^+ 14.7 ppm.

2.2 EXPERIMENTAL DESIGN

The experiment was arranged in a randomized complete block design (RCB design) with four dripline spacings and three replicates. The four dripline spacings composed of: T1 (S75) in which one dripline was used for each crop row (1:1), T2 (S150) in which one dripline served two crop rows (1:2), T3 (S225) in which one dripline served three crop rows (1:3), and T4 (S300) in which one dripline served four crop rows (1:4). Single-row production system was used, i.e., conventional plant row widths, in which corn was sown with a regular row spacing of 75 cm and spaced 20 cm apart within row, giving a plant density of about 67,000 plants ha^{-1}. Each experimental unit in S75 contained five 75 cm crop rows and five 75 cm driplines. In S150, each experimental unit contained six 75 cm crop rows and three 150 cm driplines. In S225, each experimental unit contained nine 75 cm crop rows and three 225 cm driplines. In S300, each experimental unit contained twelve 75 cm crop rows and three 300 cm driplines (Fig. 1). The length of each experimental unit was 5 m. The number of crop rows per unit area (per hectare) and, therefore, the plant density (about 67,000 plants ha^{-1}) were maintained equal for all treatments.

The field was conventionally prepared, i.e., ploughed and disked before planting. A sufficient spacing was maintained between plots to minimize water intervention among treatments. Sweet corn (*Zea mays* 'Silver Queen White') was sown on April the 18th and the 3rd in the 2017 and 2018 growing seasons, respectively. As the area was not too large, the hand sowing was used. The plant densities after establishment and at harvest were similar within the treatments. Weeds were manually removed many times during both cropping seasons. Because of the lack of rainfall and the inability of drip irrigation to germinate corn seeds especially in plots with wider dripline spacings, a secondary lateral move irrigation system (available on site) was used at the beginning of each season. It was used two times during the first month with 100 mm water applied in each growing season. After that, the drip irrigation started on as scheduled and continued until termination. Driplines (GR type, 16 mm diameter) with a built-in 40-cm emitter spacing and a nominal flow rate of 4 l hr^{-1} (10 l hr^{-1} m^{-1}) were used.

2.3 DATA MANAGEMENT

The climate data were used to calculate the reference evapotranspiration (ET_0) using the FAO Penman-Monteith equation. Sweet corn crop data, i.e., growth stages in days, crop coefficient value (*Kc*) at each growth stage, were obtained from FAO databases (FAO, 1998). The duration of the initial, development, mid-season and late season growth stages were 20, 30, 40, and 10 days, respectively. The crop coefficient (*Kc*) is basically the ratio of the crop evapotranspiration (*ETc*) to the reference evapotranspiration (ET_0), and it represents an integration of the effects of characteristics that distinguish the sweet corn crop from reference grass. *Kc* values were 0.3, 1.15, and 1.05 for initial, mid-season and late season growth stages, respectively. Deviation of the Kc in both mid-season and late season stages from the reference value '1' is mainly due to differences in crop height and resistance between the grass reference surface and the sweet corn crop and weather conditions (FAO, 1998). Daily crop evapotranspiration (crop water requirement, *ETc*) was calculated by multiplying the daily ET_0 by the crop coefficient *Kc* within each growth stage.

Crop water requirement data were used for scheduling irrigation. Corn crop was irrigated once a week, because it is usually more convenient to adjust the irrigation schedule weekly. The weekly sum of daily *ETc* values was used to adjust the schedule for the following week. The weekly adjustment ensures that soil water content in the rootzone was replenished. The same volumes of water per unit area were applied to the other treatments. In other words, the irrigation water depth applied was equal for all treatments. However, treatments differed in terms of irrigation times, as calculated by Eq. (1), due to the differences in dripline spacings.

$$\text{Irrigation time (hr)} = \frac{\text{dripline spacing (cm)} \times \text{emitter spacing (cm)} \times \text{irrigation amount (mm)}}{10000 \times \text{emitter rate } (\text{l hr}^{-1})} \qquad (1)$$

Volumetric soil water content was observed using neutron scattering technique (NP), on a weekly basis each growing season (about 72 hours after irrigation event). The probe was *in-situ* calibrated before sowing. Neutron probe access tubes were installed in the crop rows according to the distance from the dripline (Fig 1). In other words, one soil profile was tested in both S75 and S150, whereas two soil profiles were studied in both S225 and S300 according to the location of plant row relative to the dripline: (a) at the crop row close to the dripline, and (b) at the distant crop row from the dripline.

The total soil water storage (*SWS*) from the soil surface to the root zone depth (Zm = 1.2 m) were calculated by the following equation:

$$\mathrm{SWS} = \int_0^{Zm} \theta(z)\, dz \tag{2}$$

where θ is volumetric soil water content (cm^3 cm^{-3}), and z is soil depth (m). For the purpose of comparison, changes over time in *SWS* which were directly measured under plants, were jointly plotted, i.e., presented together graphically for the purposes of comparison. In order to check soil water stress under crop rows, the parameters describing the water stress response function as suggested by Feddes et al. (1978), are also used. This function defines the manner in which transpiration is reduced below potential when the soil is no longer able to supply the required amount of water by plant under the prevailing climatic conditions. This function assigns plant root water uptake rates according to the local soil water pressure head (h), at any point in the root zone. Water uptake in this model is assumed to be zero close to saturation. Water uptake is considered optimal between two specific values of pressure head h_2 and h_3. While for soil water pressure heads between h_3 and h_4, water uptake decreases. Root water uptake is also considered to be zero for soil water pressure heads less than the wilting point pressure head (more negative, $h < h_4$). The values of soil water content (θ) corresponding to h_2, h_3, and h_4 were calculated as 44.5, 23.2, and 13.6 cm^3 cm^{-3}, respectively, for sweet corn crop and for the clay loam soil, according to Feddes et al. (1978). Soil water storages corresponding to these root water uptake parameters were also presented graphically.

For each cropping season, the field was fertilized in early winter with 46.0 kg of P_2O_5 ha^{-1} as triple super phosphate (TSP). However, N-fertilizer as urea 46 % solution (150 kg N ha^{-1}) was applied in two equally split applications. The 1st was applied at sowing day, while the 2nd was one month later. Thus, all plots received the same amounts of both N and P fertilizers.

Regarding fresh marketable yield, harvest was done when corn silk began to turn brown, which occurred approximately at the milky stage with seed water content of about 70-75 % (100 days after planting for both growing seasons). A 2 m row length (10 plants) from the centre of each plot (individual replicate) was selected. For plots with wider dripline spacings (S225 and S300) two adjacent plant rows which represented the nearest and the furthest crop rows from dripline, were selected to estimate an integrated measured parameters for these treatments, and to assess the yield distribution according to the row distance from dripline. The heights of selected plants were measured from the soil surface up to the beginning of tassels. Fresh green cobs from selected plants were hand harvested. Mass (CM), length (CL), and diameter (CD) of husked cobs were measured. All cobs with good grain filling and longer than 10 cm were considered marketable cobs according to the common corn-farming practices in the area. Following the harvest of husked cobs, aboveground vegetative parts of selected plants were also harvested and oven dried at 70 ˚C until constant mass for dry matter yield determination. Mass of cobs and vegetative parts were converted into unit area yields and expressed as t ha^{-1}.

Irrigation water use efficiency (*IWUE*) was determined by dividing yield by the irrigation water amount applied (I, m^3), and expressed as kg per m^3 of water. It was calculated for husked cob yield ($IWUE_{hc}$) and dry matter yield ($IWUE_{dm}$) using equations (3 and 4), respectively.

$$\mathrm{IWUE}_{\mathrm{hc}} = \frac{\mathrm{HCY}}{\mathrm{I}} \tag{3}$$

$$\mathrm{IWUE}_{\mathrm{dm}} = \frac{\mathrm{DMY}}{\mathrm{I}} \tag{4}$$

2.4 STATISTICAL DATA ANALYSIS

The measured parameters, i.e., plant height (H), mass (CM), length (CL), and diameter (CD) of husked cobs, husked cob yield (HCY), dry matter yield (DMY), and both irrigation water use efficiencies, were subjected to an one-way analysis of variance using the DSAASTAT add-in version 2011 (Onofri, 2007). A combined analysis of data over both tested years was conducted according to Gomez and Gomez (1984), to recognize dripline spacing whose average effect over years is high and stable. Mean comparison was performed after combined analysis using the least significant difference test (*LSD*) at the 5 % level of significance.

2.5 UNCERTAINTIES AND SHORTCOMINGS

The sugar, protein, oil and starch contents in seeds were not measured, due to the unavailability of the required analysis devices at the time.

As mentioned above, this study used crop coefficient values (*Kc*) of sweet corn as obtained from FAO databases (FAO, 1998), because the values for the tested cultivar were not available.

Moreover, this study included treatments in which water was applied at full levels for the various dripline spacings. In other words, the irrigation amount was applied on a land-area basis (Eq. 1). The influence of ground cover was not taken into account for drip irrigation scheduling. In other words, a reduction factor was not applied to the *ETc* calculations. The reason was to avoid the effects of variations in plant growth between treatments, which would have led to variations in the amounts of irrigation water applied.

Due to the absence of accurate data on product prices in the local market due to the huge daily fluctuation of exchange rate, because of the predominant conditions in the country, a partial budget analysis to compare the relative economic rankings of the tested dripline spacings was not conducted. However, a limited data was provided based on the prices of today (May 2020) to economically compare the studied treatments.

3 RESULTS

3.1 IMPACT OF DRIPLINE SPACING ON INTEGRATED MEASURED TRAITS

The analysis of variance (ANOVA) indicated no significant interaction year × treatment ($p > 0.05$). Therefore, the effects of dripline spacing on the measured parameters were shown as the averages of both tested years, and summarized in Table 2. Moreover, ANOVA revealed that the measured parameters were significantly influenced by the dripline spacing at the 5 % level (Table 2).

Dripline spacing had a strong impact on the plant height, H, ($p < 0.05$). With no significant differences between S75 and S150, both treatments produced the tallest plants up to the beginning of tassels. Then, H decreased as dripline spacing increased. The smallest mean value of H was recorded under the 300-cm spacing conditions (Table 2). The maximum value of husked cob length (18.7 cm) was found under the conventional treatment, S75. Then, it significantly decreased as the dripline spacing increased. The 300 cm dripline spacing produced cobs significantly shorter by about 20 % than those in S75. No significant differences were recorded between S75 and S150, nor between S225 and S300 (Table 2). Also, dripline spacing treatments differed significantly in term of diameter of husked cobs. The largest cob diameter was produced under both 75 and 150 cm spacings (4.8 and 4.7 cm, respectively); while cobs produced under both 225 and 300 cm dripline spacings were significantly shorter by about 11 % (Table 2). With regard to the mean mass of husked cob (CM), no significant differences were

Table 2: Mean comparisons of crop responses as function of dripline spacings

Dripline spacing	H (cm)	CL (cm)	CD (cm)	CM (g cob^{-1})	DMY (t ha^{-1})	HCY (t ha^{-1})	IWUE$_{dm}$ (kg m^{-3})	IWUE$_{hc}$ (kg m^{-3})
2017 season	161.7	18.7	4.8	211.6	7.96	13.91	1.06	1.85
S75 (1:1)	164.9	16.5	4.7	254.0	7.37	11.66	0.98	1.55
S150 (1:2)	146.8	16.2	4.3	183.7	7.46	8.75	0.99	1.16
S225 (1:3)	136.1	15.5	4.3	223.4	6.24	6.41	0.83	0.85
S300 (1:4)								
2018 season								
S75 (1:1)	147.9	18.7	4.8	224.2	7.78	13.94	1.11	1.99
S150 (1:2)	155.5	18.5	4.8	181.2	7.98	12.95	1.14	1.85
S225 (1:3)	133.5	15.3	4.1	181.3	8.68	9.62	1.24	1.37
S300 (1:4)	136.7	14.8	4.1	207.8	6.89	7.32	0.98	1.05
Combined data								
S75 (1:1)	154.8 a	18.7 a	4.8 a	244.3 a	7.87 a	13.93 a	1.08 a	1.92 a
S150 (1:2)	160.2 a	17.5 a	4.7 a	217.9 a	7.68 a	12.30 b	1.06 a	1.70 b
S225 (1:3)	140.1 b	15.8 b	4.2 b	217.6 a	8.07 a	9.18 c	1.12 a	1.27 c
S300 (1:4)	136.4 b	15.1 b	4.2 b	182.5 b	6.57 b	6.86 d	0.91 b	0.95 d
LSD$_{0.05}$	5.57	1.41	0.18	34.25	0.94	1.39	0.13	0.19

In each column for combined data, means followed by different letters are significantly different according to LSD test at 5 % level.
H = plant height, CL = length of husked cob, CD = diameter of husked cob, CM = mass of husked cob, DMY = dry matter yield, HCY = husked cob yield, IWUEdm = irrigation water use efficiency for dry matter, and IWUEhc = irrigation water use efficiency for husked cobs.

recorded between S75, S150, and S225 treatments (Table 2). Whereas the 300 cm dripline spacing produced cobs significantly lighter (182.5 g cob^{-1}).

The integrated dry matter yield (DMY) varied significantly among treatments, ranging from the lowest value of 6.57 t ha^{-1} in S300 treatment to the highest of 8.07 t ha^{-1} in S225 treatment. No significant differences were detected between S75, S150, and S225 at the 5 % level (Table 2). Furthermore, mean integrated yields varied widely among treatments (Table 2). The highest value (13.93 t ha^{-1}) was found under the 75 cm spacing. After that, it significantly decreased as the dripline spacing increased. The mean integrated yields in S150, S225, and S300 were reduced by 11.7, 34.0, and 50.7 %, respectively, compared with that of the 75 cm spacing (Table 2).

As mentioned earlier, all dripline spacing treatments received at each irrigation event the same amount of water per unit area (the same irrigation water depth), and treatments differed in terms of irrigation times according to the dripline spacing. Each dripline spacing treatment received about 653 mm in 2017, and 600 mm in 2018, of drip irrigation water amounts. The times of drip irrigation events applied to S75, S150, S225, and S300 were 49, 98, 147, and 196 hr in 2017, and 45, 90, 135, and 180 hr in 2018, respectively. Consequently, the total amounts of irrigation water were for each treatment 753 mm in 2017, and 700 mm in 2018, respectively. Although the climate data for both studied years were close to the 20-year mean, the difference in irrigation water amounts applied in the two years could be due to the timing of high evapotranspiration days and to the earliness in planting day in the 2018-cropping season.

For IWUE$_{dm}$, no significant differences were recorded between S75, S150, and S225. The maximum value was observed under S225 (1.12 kg m^{-3}). The wider dripline spacing treatment (S300) significantly decreased IWUE$_{dm}$ by about 17 % relative to the other treatments (Table 2). Irrigation water use efficiency for economic

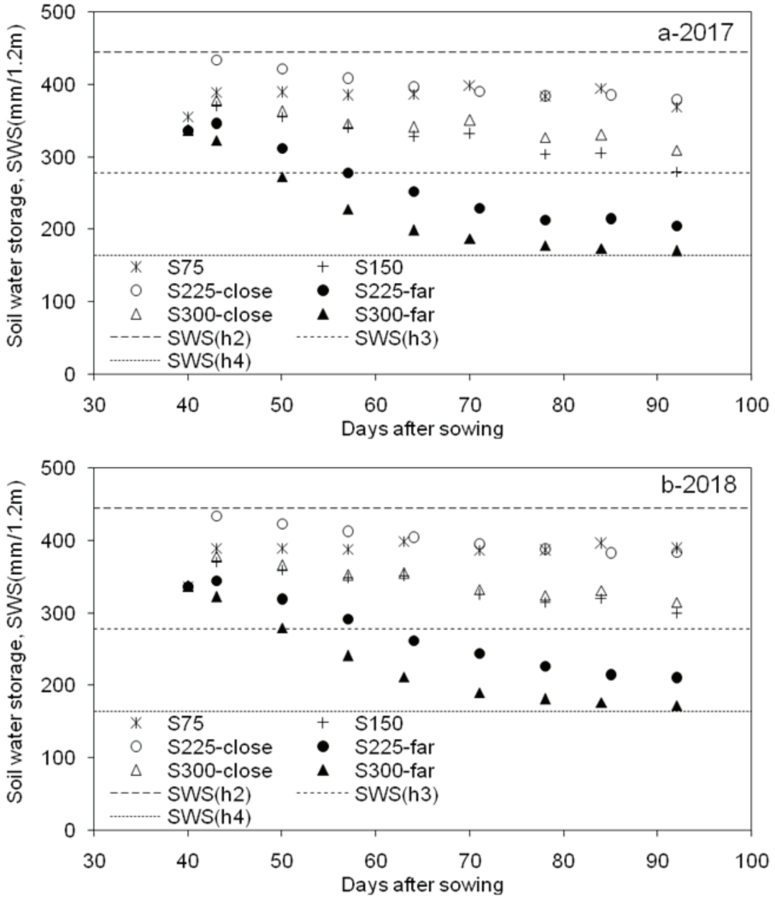


Figure 2: Changes over time in soil water storages in 120 cm soil profile for 2017 (a) and 2018 (b) growing seasons. Dashed lines represent soil water storages at *h2*, *h3*, and *h4* corresponding to the root water uptake parameters for sweet corn crop as suggested by Feddes et al. (1978). S75, S150, S225, and S300 represent dripline spacings treatments. “close” and “far” represent the row next to the dripline and the distant row from the dripline, respectively.

yield (husked cob yield), IWUE$_{hc}$, significantly decreased as dripline spacing increased. The highest value was observed under S75 (1.92 kg m^{-3}). IWUE$_{hc}$ under S150, S225, S300 were, respectively, 11.7, 34.0, and 50.7 % less than that under S75, which followed the reduction percentage trend of HCY (Table 2).

3.2 IMPACT OF DRIPLINE SPACING ON SOIL WATER STATUS

Variations over time in weekly measured soil water storages (SWS) are shown in Figure 2 for both growing seasons. Soil water storages at the rows close to driplines varied from one treatment to another, but still remained between SWS (h_2) and SWS (h_3), where root water uptake is maximal according to Feddes et al. (1978). This indicated that no water stress throughout the whole growing season was exposed on the corn plants (Figs. 2 a and b). This can be attributed to the fact that the crop rows closest to driplines have more favorable soil water conditions. However, in both growing seasons, irrigation maintained the soil water storage at a fairly constant and high level under the conventional 75 cm dripline spacing relative to the other treatments. This indicated that the 75 cm spacing was adequately providing crop water requirements.

When using wider dripline spacings, as for both S225 and S300 treatments, soil water storages remarkably decreased at the distant crop rows from driplines. Their values soon dropped below the lower limit of optimal range of root water uptake, SWS (h_3), below which roots can no longer extract water at the maximum rate, and the transpiration was reduced below potential rate (Feddes et al., 1978). Thus, the distant rows for both treatments S225 and S300 were susceptible to water stress starting from the end of development stages (about 50 and 60 days after sowing day for S300 and S225, respectively). The degree of water stress excessively increased during the reproductive stages towards the late stage. However, their values did not drop below the lowest limit SWS (h_4), below which root water uptake was zero reaching the wilting point pressure head.

3.3 IMPACT OF DRIPLINE SPACING ON INDIVIDUAL CROP ROW YIELDS

The effects of dripline spacing on individual crop row yields are illustrated in Figure 3 for husked cob yield and in Figure 4 for dry matter yield. In order to show the yield distributions of the different treatments, a mirror image of yields versus a dripline was used. Large yield differences between rows took place as distance from the dripline to the crop row increased. Both HCY and DMY increased as the crop rows were moved closer to the driplines. In the 300-cm dripline spacing, the husked cob yield (HCY) for the interior rows (rows close to the dripline) was 94.5 % higher than for the exterior rows (rows far away from the dripline). Moreover, the dry matter yield (DMY) for the interior rows was 44.5 % higher than for the exterior rows. However, they were 60.6 % for HCY and only 18.7 % for DMY under the 225-cm spacing conditions (Figs. 3 and 4).

On the other hand, for the same distance of crop row from dripline, expected results occurred under both wider dripline spacings (S225 and S300) conditions com-

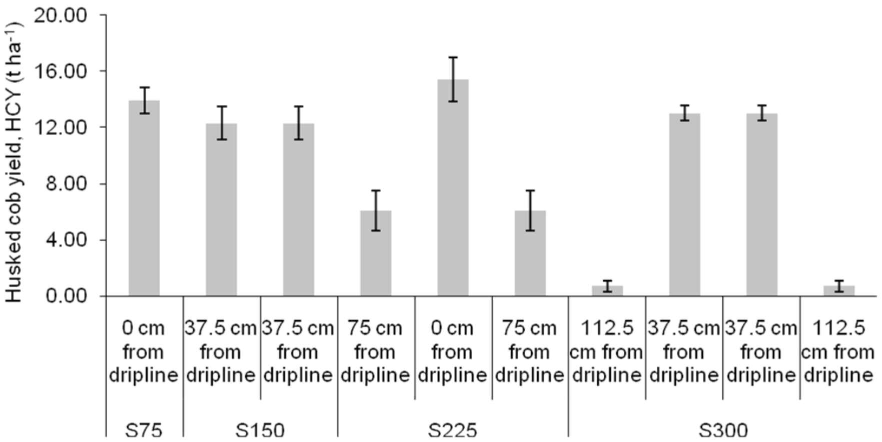


Figure 3: Husked cob yield distribution according to the distance from dripline for the combined data of both tested seasons. Individual crop row yields are mirrored about the dripline in each treatment for display purposes. S75, S150, S225, and S300 represent dripline spacing treatments. Error bars represent the standard deviations.

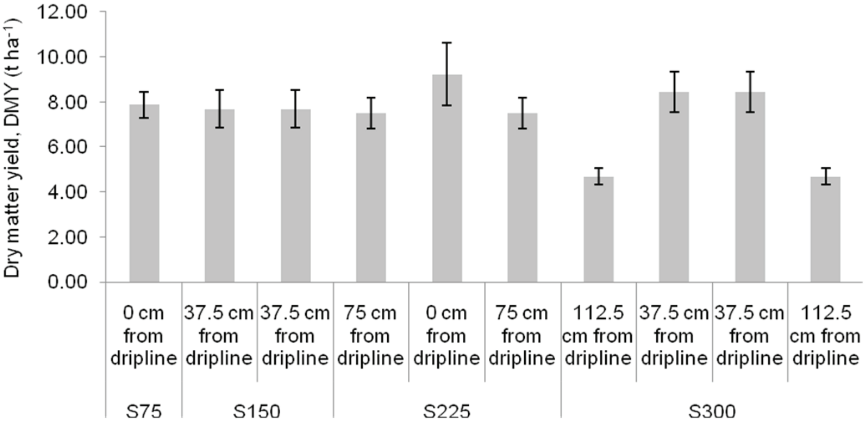


Figure 4: Dry matter yield distribution according to the distance from dripline for the combined data of both tested seasons. Individual crop row yields are mirrored about the dripline in each treatment for display purposes. S75, S150, S225, and S300 represent dripline spacing treatments. Error bars represent the standard deviations.

pared with S75 and S150 conditions. For the crop rows placed at 37.5 cm from dripline as in both S150 and S300 treatments, the husked cob yields were somewhat similar (12.30 and 13.02 t ha^{-1} in S150 and S300, respectively). While for the crop rows placed at 0 cm from dripline as in both S75 and S225 treatments, the husked cob yield in S225 (the interior rows) (15.41 t ha^{-1}) was 10 % higher than their analogues under S75 (13.93 t ha^{-1}) (Fig. 3). In addition, the dry matter yields for the interior rows in both S225 and S300 were almost higher than those under both S75 and S150, respectively (Fig. 4).

4 DISCUSSION

As different dripline spacings were tested, one dripline served one or several plant rows, and therefore, rootzone soil water content was spatially variable and depended on the distance from the dripline. Results indicated insufficient supply of irrigation water to the points of rootzone very distant from dripline, and consequently, water distribution was inappropriate for the wider dripline spacings, even with the same amount of irrigation water applied. This result is in agreement with other published findings by Lamm et al. (1997) who found that soil water storages were reduced an additional 20 to 40 % by the crop rows placed away from the driplines at 114 cm, relative to those at crop rows growing next to driplines at 38 cm. They demonstrated that water supply was improper for the wider dripline spacings, even with full irrigation. Bozkurt et al. (2006) assessed also the effects of different dripline spacings (70, 140, and 210 cm) on yield and yield components of corn crop, and reported that insufficient irrigation water was supplied to crops away from the dripline, even under full irrigation. This water stress was gradually increased under deficit irrigation conditions especially in the wider spacing treatments.

The row-to-row yield variation was enormous under the 300-cm spacing conditions, and less sharply under 225 cm spacing treatment. This row-to-row variation was attributed to the spatial variation in rootzone soil water status. In plots with the 225 and 300 cm dripline spacings, the crop rows in the immediate vicinity of the dripline received a higher water amount than those away from the dripline. As mentioned above, the furthest crop rows were more water stressed than rows adjacent to the dripline. Thus, yields of the interior crop rows (next to the driplines) in 225 and 300 cm spacings were found higher or equal to those of 75 and 150 cm spacings. This could be due to the extra water available for the interior rows in the wider dripline spacings, mitigating the severity of wetting-drying cycle between two irrigation events. Moreover, this more favourable soil water condition may stimulate plants to consume more nutrients. Another possibility is that the furthest crop rows from dripline were short in height due to the soil water stress (data not shown). This could allow more sunlight into the crop canopy of the interior rows, resulting in higher yields (Dalley et al., 2004; Bradley, 2006). This is in agreement with several experimental results which found that non-uniformity of yield with the distance from the dripline negatively affects the total yield. Lamm et al. (1997) reported significant effect of row spacing across dripline on corn yield under subsurface drip irrigation system, and documented that the row-to-row variation could reach more than 67 and 41 % for 230 and 300 cm dripline spacings, respectively. These yield reductions could attain 95 % in both dripline spacings under deficit irrigation conditions. However, Bozkurt et al. (2006) reported that yield from the wider dripline spacing (210 cm) with full irrigation for the closest and furthest crop rows from the dripline was only 8.2 and 9.6 % lower, respectively, than that of the optimum dripline spacing (140 cm). These reductions in yields reached 25.9 and 36.9 %, respectively, when irrigation level reduced to 67 % of crop water needs.

Results indicated that rootzone soil water status enhanced as the plant rows were moved closer to the driplines. This was reflected in the integrated traits, i.e., plant height, ear characteristics, yields, and irrigation water use efficiency. They were found to be decreased as the dripline spacing increased. Similar results for husked cob length, diameter, and mass were reported by previous studies (e.g. Al-hurmuzi and Topak, 2018; Mubarak, 2020a and b). However, Bozkurt et al. (2006) reported that the effects of different dripline spacings on cob length were statistically insignificant, but cob length was reasonably higher in full irrigation relative to the deficit irrigation condition. Similar yields to those obtained herein were also found by Ertek and Kara (2013) when a dripline per crop row was used. They reported that the fresh cob yield of sweet corn reached 14.74 t ha^{-1}. A fresh cob yield of sweet corn of 13.43 t ha^{-1} was also reported by Oktem et al. (2003). Basava (2012) studied the effects of one dripline for two sweet corn crop rows at 120 cm, and reported a fresh cob yield of 12.83 t ha^{-1} under full irrigation level. Lamm et al. (1997) showed that mean corn yields were 13.6, 12.8, and 12.2 t ha^{-1} for 150, 230, and 300 cm dripline spacings, respectively, for a total irrigation volume of 462 mm, using subsurface drip irrigation. Corn yields reduced to 10.8 and 9.3 t ha^{-1} when irrigation level was decreased by 33 and 50 % for the wider dripline spacings of 230 and 300 cm, respectively. However, Sorensen and Lamb (2008) found that corn yield showed no difference between 91 and 183 cm dripline spacings. Moreover, Bozkurt et al. (2006) reported that the mean corn yields were 8.57, 9.79, and 8.92 t ha^{-1} for 70, 140, and 210 cm dripline spacings, respectively for a full irrigation with a total amount of about 750 mm, using surface drip irrigation. Different from our findings, those authors documented that the wider dripline spacing (140 cm) was better than the closer dripline spacing (70 cm) under full irrigation.

A partial budget analysis should have been conducted to compare the relative economic rankings of the tested dripline spacings. A limited data could be provided based on the prices of today (May 2020). The local price of sweet corn is about 150 USD per tonne. The cost of dripline plus the cost of its laying out on the field is about 0.30 USD per meter. As mentioned above, HCYs were 13.93, 12.30, 9.18 and 6.86 t ha^{-1} in S75, S150, S225 and S300, respectively (Table 2). The total incomes from the harvested husked cobs were about 2090, 1845, 1377 and 1029 USD per year, respectively. As well, the initial costs of driplines were about 4000, 2000, 1333 and 1000 USD per hectare, respectively. Hence, the production of two growing seasons under S75 conditions (the 75 cm spacing) would be needed in order to recover the initial costs of driplines, assuming the consistency of yield over years. However, the production of only one season would be needed to recover the initial costs in the wider spacings.

The S150, S225 and S300 produced, respectively, about 1.63, 4.75 and 7.07 t ha^{-1} less than S75. This means a loss of 245, 713 and 1061 USD per hectare, respectively, as compared with S75. However, the wider dripline spacing, the higher initial unit-area dripline cost savings, attaining 50, 66.7, and 75 % for the 150, 225, and 300 cm spacings, respectively, relative to that in the conventional 75 cm dripline spacing. In other words, the initial unit-area dripline cost savings are 2000, 2667 and 3000 USD per hectare, respectively. Hence, the reduction in crop yield due to the wider dripline spacings did not justify the extra cost of the closer dripline spacing. This result agreed well with the findings of other studies (Lamm et al., 1997; Bozkurt et al. 2006).

Moreover, reducing the quantity of driplines in unit area according to the dripline spacings reveals decreases in terms of main pipeline diameter, related accessories and pumping requirements, from the point of view of irrigation network design. Moreover, it reduces the harmful environmental impacts resulting from broken plastic components. Although the irrigation times was 2, 3, and 4 times as much of S75 treatment, when using 150, 225, and 300 cm dripline spacings, respectively, the decreases in the pump unit capacity probably compensate the total electric energy needed over the growing season in comparison to the other treatments, also from a design perspective.

Otherwise, results of a simulation study using Hydrus2D code performed on the herein tested treatments (data not shown) demonstrated that an important portion of water amounts provided by irrigation were lost by deep percolation, when using the wider lateral spacing. The highest deep-percolated volume was occurred under S300 conditions, with more than 10 % of the total amount of irrigation water were deep-percolated under the 150 cm soil depth. The deep percolation of irrigation water which carries much of the mobile nutrients can adversely affect the environment and the groundwater quality. This reveals that the 300 cm dripline spacing is not suitable for the environmental protection due to deep percolation losses under the study context.

The maximum two-year average husked cob yield was observed under 75 cm dripline spacing, and then it significantly decreased as dripline spacing increased as above mentioned. Some farmers may find the wider dripline spacings not suitable for the drip-irrigated sweet corn production due to long irrigation set times, non-uniformity of the corn field scene, and probable deep percolation losses. Nevertheless, although the yield reductions under wider dripline spacings involved economically considerable losses at the high overall levels, they did not justify the extra cost of the closer dripline spacing, at very low crop prices and very high dripline costs.

5 CONCLUSION

Increasing the spacing between driplines has been recognized as the most significant factor in reducing the high initial costs of drip irrigation system. Nevertheless, almost none research findings are presently available on sweet corn response to the same tested planting arrangement (i.e., using one dripline for three or four crop rows of sweet corn) grown in the dry Mediterranean area. The 75 cm dripline spacing conditions produced the maximum two-year average husked cob yield (13.93 t ha^{-1}), and the irrigation water use efficiency (1.92 kg m^{-3}). Although the 150, 225 and 300 cm spacings yielded, respectively, 11.7, 34.0 and 50.8 % less than the 75 cm dripline spacing, these wider dripline spacings provided 50, 67 and 75 % less unit-area dripline cost, respectively. These reductions in crop yield did not justify the extra cost of the closer dripline spacing. The production of two seasons would be needed to recover the initial dripline costs under closer dripline spacing. However, the production of only one season would be needed for the wider spacings. Hence, using wider dripline spacings would be economically justified at low crop prices and high dripline costs.

The sugar, protein, oil and starch contents in seeds were not measured, while economic yields and irrigation water use efficiency and some plant and cob characteristics were presented. This study used crop coefficient values (*Kc*) of sweet corn as obtained from FAO databases. Moreover, this study included treatments in which water was applied at full levels, so that the irrigation amount was applied on a land-area basis. The influence of ground cover was not taken into account for drip irrigation scheduling in order to avoid the effects of variations in plant growth between treatments, which would have led to variations in the amounts of irrigation water applied. A limited data was provided based on the prices of today to economically compare the studied treatments, due to the absence of accurate data on product prices in the local market because of the predominant conditions in the country.

The outcomes of this study were conveyed to the policymakers (the Ministry of Agriculture and Agrarian Reform) in order to adopt the practical alternatives based on the obtained results and to make the wider dripline spacings in sweet corn crop production system familiar for most farmers. It is requested to stimulate farmers to adopt the proposed alternatives in their fields to sustain corn productivity, to reduce the high initial costs of drip irrigation system.

For the scientific community, our findings demonstrated that wide dripline spacings resulted in less driplines in unit area, less related accessories and fewer requirements of pump unit capacity. This may contribute to introduce practical alternative to meet sustainable crop production, water shortage, and environmental protection in the dry Mediterranean region.

Finally, further studies are needed in order to benefit from the higher initial unit-area dripline cost savings obtained under wider spacings. Adopting suitable techniques, such as using multiple-row production systems, may enhance soil water status and root water uptake, and therefore, final fresh yield. Moreover, further studies should include treatments in which water was applied at reduced levels (not only at full levels as in this study) for the various spacings, in order to determine if the irrigation amount should be applied on a land-area basis (full) or in relation to number of driplines in plots (reduced). The fraction of the total surface area actually covered by the foliage of the plants (ground cover) should be also applied for irrigation scheduling under drip irrigation. Moreover, it is recommended to study the influences of tested planting arrangement on sugar, protein, oil and starch contents in seeds.

6 ACKNOWLEDGEMENT

The authors would like to thank the Atomic Energy Commission of Syria for encouragement and support.

7 REFERENCES

Al-hurmuzi, J. O. H., Topak, R. (2018). Lateral spacing affect on yield and quality attributes of drip-irrigated sweet corn. *Selcuk Journal of Agricultural and Food Sciences, 32*(1), 55-59. https://doi.org/10.15316/SJAFS.2018.64

Basava, S. (2012). Performance of sweet corn hybrid under different levels of irrigation and nitrogen applied through drip system. Master of Science in Agriculture, Acharya N. G. Ranga Agricultural University, Hyderabad.

Bordovsky, J. P., Porter, D. (2003). Cotton response to preplant irrigation level and irrigation capacity using spray, LEPA, and subsurface drip irrigation. ASAE Paper No. 032008. St. Joseph, Mich.: *American Society of Agricultural Engineers*.

Bosch D.J., Powell N.L., Wright F.S. (1998). Investment returns from three subsurface microirrigation tubing spacings. *Journal of Production Agriculture, 11*(3), 371-376. https://doi.org/10.2134/jpa1998.0371

Bozkurt, Y., Yazar, A., Gencel, B., Sezen, M. S. (2006). Optimum lateral spacing for drip-irrigated corn in the Mediterranean Region of Turkey. *Agricultural Water Management, 85*, 113-120. https://doi.org/10.1016/j.agwat.2006.03.019

Bradley, K. W. (2006). A review of the effects of row spacing on weed management in corn and soybean. *Crop Management*. https://doi.org/10.1094/CM-2006-0227-02-RV

Camp, C. R., Sadler, E. J., Busscher, W. J. (1989). Subsurface and alternate-middle microirrigation for the southeastern Coastal Plain. *Transactions of the American Society of Agricultural Engineers, 32*(2), 451-456. https://doi.org/10.13031/2013.31025

Camp, C. R., Bauer, P. J., Hunt, P. G. (1997). Subsurface drip irrigation lateral spacing and management for cotton in the southeastern Coastal Plain. *Transactions of the American Society of Agricultural Engineers, 40*(4), 993-999. https://doi.org/10.13031/2013.21351

Camp, C. R. (1998). Subsurface drip irrigation: a review. *Transactions of the American Society of Agricultural Engineers, 41*(5), 1353-1367. https://doi.org/10.13031/2013.17309

Charlesworth, P. B., Christen, E. W., de Vries, T. (1998). Is S.I.P. (Draincoil) a cheap alternative to drip irrigation? In Water is Gold: National Conference and Exhibition Proceeding, 499-506.

Charlesworth, P. B., Muirhead, W. A. (2003). Crop establishment using subsurface drip irrigation: A comparison of point and area sources. *Irrigation Science, 22*(3-4), 171-176. https://doi.org/10.1007/s00271-003-0082-6

Clemmens, A. J., (2002). Measuring and improving irrigation performance at the field level. *Transactions of the American Society of Agricultural Engineers, 22*(8), 89-96.

Dalley, C. B., Kells, J. J., Renner, K. A. (2004). Effect of glyphosate application timing and row spacing on weed growth in corn (*Zea mays*) and soybean (*Glycine max*). *Weed Technology, 18*, 177-182. https://doi.org/10.1614/02-150B

Ertek, A., Kara, B. (2013). Yield and quality of sweet corn under deficit irrigation. *Agricultural Water Management, 129*, 138-144. https://doi.org/10.1016/j.agwat.2013.07.012

FAO. (1998). *Crop evapotranspiration: guidelines for computing crop requirements* (FAO Irrigation and Drainage Paper No. 56).

Feddes, R. A., Kowalik, P. J., Zaradny, H. (1978). *Simulation of Field Water Use and Crop Yield*. Wiley, New York.

Gomez, K. A., Gomez, A. A. (1984). *Statistical Procedures for Agricultural Research* (2nd ed.). New York, NY: John Wiley & Sons.

Henggeler, J. C. (1995). A history of drip-irrigated cotton in Texas. In Microirrigation for a Changing World: Conserving Resources/Preserving the Environment, *Proceeding. 5th International Microirrigation Congress, 669–674. F.* R. Lamm, ed. St. Joseph, Mich.: American Society of Agricultural Engineers.

Howell, T. A., Schneider, A. D., Evett, S. D. (1997). Subsurface and surface microirrigation of corn-Southern High Plains. *Transactions of the American Society of Agricultural Engineers, 40*(3), 635-641. https://doi.org/10.13031/2013.21322

Kruse, E. G., Israeli, I. (1987). Evaluation of a subsurface drip irrigation system. *Presented at the 1987 Summer Meeting of the American Society of Agricultural Engineers*. ASAE Paper No. 87-2034. American Society of Agricultural Engineers, St. Joseph, Michigan, 21 pp.

Lamm, F. R., Stone, L. R. Manges, H. L., O'Brien, D. M. (1997). Optimum lateral spacing for subsurface drip-irrigated corn. *Transactions of the American Society of Agricultural Engineers, 40*(4), 1021-1027. https://doi.org/10.13031/2013.21354

Lamm, F. R., Trooien, T. P., (2003). Subsurface drip irrigation for corn production: A review of 10 years of research in Kansas. *Irrigation Science, 22*(3-4), 195-200. https://doi.org/10.1007/s00271-003-0085-3

Mubarak, I., (2020)a. Triple-row system with a wider drip-line lateral spacing for two drip-irrigated sweet corn cultivars. *Pesquisa Agropecuaria Brasileira,* 55, e01684. https://doi.org/10.1590/S1678-3921.pab2020.v55.01684. 2020.

Mubarak, I., (2020)b. The response of two drip-irrigated sweet corn varieties to the twin-row production system in the dry Mediterranean region. *The Open Agriculture Journal*, *14*, 9-15. https://doi.org/10.2174/1874331502014010009

Oktem, A., Simsek, M., Oktem, A. G. (2003). Deficit irrigation effects on sweet corn with drip irrigation system in semi arid region: I. water-yield relationship. *Agricultural Water Management, 61*, 63-74. https://doi.org/10.1016/S0378-3774(02)00161-0

Onofri, A. (2007). Routine statistical analyses of field experiments by using an Excel extension. National Conference Italian Biometric Society. Proceeding 6th (Pisa). In: "*La statistica nelle scienze della vita e dell'ambiente*"; 20-22, 93-96.

Powell, N. L., Wright, F. S. (1993). Grain yield of subsurface microirrigated corn as affected by irrigation line spacing. *Agronomy Journal, 85*(6), 1164-1169. https://doi.org/10.2134/agronj1993.00021962008500060014x

Sorensen, R. B., Lamb, M. C. (2008). Corn and cotton yield with two surface drip lateral spacings. *Crop Management,* https://doi.org/10.1094/CM-2008-0118-01-RS

Spurgeon, W. E., Manges, H. L. (1990). Drip line spacing and plant population for corn. In: *Proceeding of the Third National Irrigation Symposium, Phoenix, Arizona, October 28-November 1, 1990*, pp. 217-222.

Thorburn, P. J., Cook, F. J., Bristow, K. L. (2003). Soil-dependent wetting from trickle emitters: implications for trickle design and management. *Irrigation Science, 22*, 121-127. https://doi.org/10.1007/s00271-003-0077-3

Tiercelin, J. R., (2007). *Traité d'irrigation*. Lavoisier Tec&Doc Edition.

Zhou, L. F., Feng, H., Zhao, Y., Qi, Z. J., Zhang, T. B., He, J. Q., Dyck, M. (2017). Drip irrigation lateral spacing and mulching affects the wetting pattern, shoot-root regulation, and yield of maize in a sand-layered soil. *Agricultural Water Management, 184*, 114-123. https://doi.org/10.1016/j.agwat.2017.01.008

doi:10.14720/aas.2020.116.1.1602 Original research article / izvirni znanstveni članek

Efficacy and economics of integrated weed management in groundnut (*Arachis hypogea* L.)

Oludamilola ADEMABAYOJE [1], Joseph ADIGUN [1], Olusegun ADEYEMI [1], Olumide DARAMOLA [1, 2], Godwin AJIBOYE[3]

Received April 8, 2020, accepted August 12, 2020.
Delo je prispelo 08. aprila 2020, sprejeto 12. avgusta 2020.

Efficacy and economics of integrated weed management in groundnut (*Arachis hypogea* L.)

Abstract: Weed management is an important and expensive step in groundnut production. Field experiments were conducted in the early and late wet seasons of 2017 to evaluate the effectiveness and profitability of weed management using hoe weeding, herbicides or their combination in groundnut production. Butachlor and propaben at 2.0 kg a.i (active ingredient) ha^{-1} each followed by (fb) supplementary hoe-weeding (shw) at 6 weeks after sowing (WAS) significantly reduced weed cover and biomass with subsequent increase in groundnut pod yield similar to hoe-weeding treatments and better than either herbicide applied alone. The highest groundnut pod yield (1485.7 kg ha^{-1}) and revenue ($1639.2) in the early season was obtained with three hoe weeding passes. However, in the late season, the highest groundnut pod yield (1146.3 kg ha^{-1}) was obtained with propaben plus hoe-weeding and the highest revenue ($1264.8) obtained with butachlor plus hoe-weeding. Although three hoe-weedings gave the highest revenue in the early season, the gross margin and cost-benefit ratio obtained with hoe weeding treatments was lower than those of herbicides fb shw. This study showed that integrated weed management with butachlor or propaben and fb shw will improve weed control, productivity and profitability of groundnut production. Multiple hoe weeding, however, did not guarantee the highest profit but rather increased the cost of production.

Key words: butachlor; economics; efficacy; herbicides; hoeing; integrated weed management; propaben

Učinkovitost in ekonomičnost integriranega upravljanja s pleveli pri pridelavi arašidov (*Arachis hypogea* L.)

Izvleček: Upravljanje s pleveli je pomemben in drag korak pri pridelavi arašidov. V ta namen je bil izveden poljski poskus v zgodnji in pozni deževni dobi 2017 za ovrednotenje učinkovitosti in donosnosti upravljanja s pleveli v pridelavi arašidov z okopavanjem, herbicidi in njuno kombinacijo. Uporabi butaklora in propabena pri 2,0 kg (aktivne snovi) ha^{-1} je v obeh primerih sledilo še dodatno okopavanje 6 tednov po setvi, kar je značilno zmanjšalo pokrovnost in maso plevelov s povečanjem pridelka strokov arašidov, ki je bil podoben tistemu, kjer je bilo samo okopavanje, a boljši kot pri uporabi samo katerega od herbicidov. Največji pridelek strokov arašidov (1485,7 kg ha^{-1}) in donos ($1639,2) je bil v zgodnji sezoni dosežen s tremi okopavanji. V pozni sezoni je bil največji pridelek strokov arašidov (1146,3 kg ha^{-1}) dosežen z uporabo propabena z dodatnim okopavanjem in največji donos ($1264,8) pri uporabi butaklora z dodatnim okopavanjem. Čeprav so dala trikratna ročna okopavanja največji donos v zgodnji sezoni sta bila neto dobiček in razmerje stroškov in dohodkov pri okopavanju manjša kot pri uporabi herbicidov z dodatnim okopavanjem. Raziskava je pokazala, da bi pri integriranem uravnavanju plevelov z butaklorom ali s propabenom dodatno okopavanje izboljšalo nadzor nad pleveli, povečalo pridelavo in donosnost pridelave arašidov. Večkratno zatiranje plevelov samo z okopavanjem ni zagotovilo večjega dobička ampak je povečalo stroške pridelave.

Ključne besede: butaklor; ekonomičnost; učinkovitost; herbicidi; okopavanje; integrirano upravljanje s pleveli; propaben

---

[1] Federal University of Agriculture Abeokuta, Department of Plant Physiology and Crop production
[2] Corresponding author, e-mail: olumidedara01@gmail.com
[3] Federal University of Agriculture Abeokuta, Department of Soil Science

1 INTRODUCTION

Groundnut (*Arachis hypogea* L.) is one of the most important grain legume and oilseed crops of tropical and semiarid tropical countries, where it provides a major source of edible oil and vegetable protein (Sogut et al., 2016). The kernels are rich in oil (48–50 %) and protein (25–28 %), and are source of several vitamins, minerals, antioxidants, biologically active polyphenols, flavonoids, and isoflavones (Janila et al., 2013). It provides income and livelihoods to the farmers, nutritious fodder (haulms) to livestock, and contributes to nutrition of farm families through consumption of energy- and protein-rich groundnut kernels (Birthal et al., 2011). In addition, groundnut fixes atmospheric nitrogen for its own use and the benefit of intercropped cereals and subsequent crops in rotation (Bado at al., 2006). This makes it an important crop for soil fertility improvement especially for smallholder farmers who are often unable to afford synthetic nitrogen fertilizers.

Groundnut is grown on 27.7 million ha in the world with production of about 44 million tons. Nigeria, with about 3 million tons' production is the largest producer of groundnut in Africa, and the third largest producer in the world after China (16.7 million tons) and India (6.9 million tons) (FAOSTAT, 2018). Despite the increased importance of groundnut, yield obtained from farmer's field in Nigeria and other parts of Africa are very low. In general, groundnut productivity in Africa has the lowest average yield (902.6 kg ha^{-1}) compared to America (3381.4 kg ha^{-1}), Asia (2186.8 kg ha^{-1}), Oceania (1947.3 kg ha^{-1}), Europe (3102.1 kg ha^{-1}), and the global average yield (1590.1 kg ha^{-1}) (FAOSTAT, 2018). Several biotic, abiotic and socio-economic constraints are responsible for the low yield of groundnut in Africa. Among biotic factors, weed interference is the most deleterious, causing yield reduction from the range of 13 to 100 % depending on the season, cultivars, weed composition and duration of crop-weed competition (Adigun et al., 2016; Ojelade et al., 2018). Weeds exert severe competition for nutrients, water and light, hinder pegging, compete for underground space, and make harvesting of groundnut cumbersome (Jat et al., 2011).

Hoe weeding is the predominant weed control method commonly used by farmers in Nigeria (Imoloame, 2014; Daramola et al., 2020). Although hoe weeding is very important when trying to avoid the development of potential serious weed problems, its efficacy is often compromised by the continued wet condition characteristic of the beginning of the rainy season. Hoe weeding under wet condition often causes weed to re-root and reestablish, necessitating several rounds of weeding to keep the crop weed-free and avert yield losses. This is however, tedious, inefficient, time consuming and associated with high labour demands (Datta et al., 2017; Adigun et al., 2018). In addition, labour for manual weeding is scarce and often too expensive for the average farmer to afford (Adigun et al., 2017).

Alternatively, the use of herbicides is fast and quite effective in suppressing weeds if used at the proper rate and time (Chauhan et al., 2012). Controlling weeds using herbicides reduces drudgery, protect crops from early weed competition and may minimize labour cost (Adigun et al., 2018). However, a single herbicide application may not control the entire weed spectrum with diverse physiology, morphology and time of emergence (Chauhan et al., 2012; Bhagirath et al., 2013). Herbicides efficacy is further limited under conditions of high rainfall and prolonged weed germination period (Daramola et al., 2019). Moreover, indiscriminate use of herbicide can result in unintended adverse consequences such as toxicity to off-site or non-target flora and fauna, declining species diversity and herbicide resistance (Chauhan et al., 2012; Adigun et al., 2018). Hence, the need to combine two or more weed management components for broad spectrum weed control.

Combination of reduced number of hoe-weeding passes and/or herbicide applications within the context of integrated weed management could help to improve weed control efficiency, reduce the high cost associated with multiple hoe-weeding or herbicide applications and increase groundnut yield. Although few studies (Adigun et al. 2017; Daramola et all., 2020) have reported increased weed control efficiency and higher yields with integrated weed management, economic consideration, particularly profit is more important to farmers in driving the adoption of agricultural innovation (Pannell et al. 2006). It has also been reported that practice with the best yield may not necessarily translate to the best economic benefit to farmers (Sepat et al. 2017). Hence, this study was conducted to evaluate the efficacy and economic performance of weed management methods using hoe weeding, herbicides or their combination in groundnut production.

2 MATERIALS AND METHODS

Two field studies were carried out at the University of Agriculture Abeokuta at latitude 7° 15′ N and longitude 3° 25 ′E in south western Nigeria during the early (June – September) and late (August – November) seasons of 2017. During this period, total rainfall was 538 and 431 mm, minimum temperature was 22.1 and 19.0 °C and maximum temperature was 25.0 and 29.0 °C, in the early and late seasons, respectively. The soil of the sites

Table 1: Average variable cost of groundnut cultivation in the early and late seasons of 2017

Treatments	Variable cost of cultivation (US\$ ha^{-1})^{a}											
	Early season						Late season					
	Seed/seed treatment	Land preparation	Planting	Weed control	Harvesting	Total	Seed/seed treatment	Land preparation	Planting	Weed control	Harvesting	Total
Propaben	30.0	77.1	93.9	40.4	91.9	330.3	30.0	55.2	93.9	40.4	92.9	312.4
Propaben + shw^{b}	30.0	77.1	93.9	123.4	91.9	416.3	30.0	55.2	93.9	150.0	92.9	374.4
Butachlor	30.0	77.1	93.9	45.0	91.9	337.9	30.0	55.2	93.9	45.0	92.9	317.0
Butachlor + shw^{b}	30.0	77.1	93.9	128.0	91.9	420.9	30.0	55.2	93.9	160.0	92.9	379.0
Hw^{c} twice	30.0	77.1	93.9	165	91.9	457.9	30.0	55.2	93.9	300.4	92.9	395.0
Hw^{c} thrice	30.0	77.1	93.9	248	91.9	540.9	30.0	55.2	93.9	500.1	92.9	457.0
Weedy check	30.0	77.1	93.9	0.0	91.9	292.9	30.0	55.2	93.9	0.0	92.9	272.0

Cost of cultivation are values in US dollar ha-1, aNigeria Naira was converted to US dollars at exchange rate of 362.53 Naira to US\$1.00, bshw – supplementary hoe weeding, ^{c}Hw – hoe weeding

was sandy loam with pH of 6.9 and 7.1, total nitrogen of 0.23 and 0.21 % and organic matter of 2.3 and 2.2 % in the early and late seasons, respectively. The study sites was cleared manually while ploughing and harrowing were done mechanically at two weeks' interval.

The study consisted of six weed management methods viz: butachlor at 2 kg a.i (active ingredient) ha^{-1}; butachlor at 2 kg a.i ha^{-1} followed by (fb) supplementary hoe weeding (shw) at 6 weeks after sowing (WAS); propaben at 2 kg a.i ha^{-1}; propaben at 2 kg a.i ha^{-1} fb shw at 6 WAS; hoe weeding twice at 3 and 6 WAS; hoe weeding thrice at 3, 6 and 9 WAS and the weedy check where no weed removal was done throughout the period of crop growth. Butachlor and propaben were applied pre-emergence one day after the groundnut seeds were sown. Data on weed cover score and dry weed biomass (g m^{-2}) were taken at 12 WAS. Weed cover score was accessed by visual estimate based on a scale of 0-10: where 0 represents no weed growth and 10 represents complete weed cover (Kercher et al., 2003; Tunku et al., 2007). Weed cover is usually referred to as the area of ground covered by weeds or the relative proportion of weed coverage within the plot when viewed from above (Nikoa et al., 2015). Weeds were sampled from a 50 cm^{2} quadrat randomly placed at three spots within the plots. The sampled weeds were oven-dried at 70 °C until constant mass was reached, and the resulting weight recorded in g m^{-2}.

Groundnut pods were harvested from each plot when the leaves had turned brown in both seasons. The cumulative pod mass in kg plot^{-1} were then expressed in kg ha^{-1}. Statistical analysis (ANOVA) was done with GENSTAT package while Least Significant Difference (LSD at $p \leq 0.05$) was used to separate the treatment means. Prevailing market prices of all inputs and cost of labour in Nigeria in the early and late seasons of 2017 were used to evaluate the cost of groundnut cultivation (Table 1). The cost of propaben and butachlor each at 2.0 kg a.i ha^{-1} was \$23.8 and \$28.4, respectively in the early and late seasons. Application of each herbicide required 3 mandays ha^{-1} at the cost of \$16.6 in both early and late season. In the early season, hoe weeding once, twice and thrice required 15, 30 and 45 mandays ha^{-1} at \$83, \$165 and \$248, respectively, considering the wages of \$5.5 per manday. However, in the late season it was at the cost of \$62, \$123 and \$185, respectively, considering the wages of \$4.1 per manday (Table 1). Cost of seed, sowing, land preparation, planting and harvesting were the same across all the weed management treatments (Table 1). Revenue from each weed management method was the product of groundnut pod yield (kg) and steady market price (\$1.1 kg^{-1}). The gross margin was used to determine the profitability of groundnut under the different weed management methods. The gross margin is usually referred to as returns over variable cost and services as a proxy measure of profitability (Maurice et al. 2005).

3 RESULTS AND DISCUSSION

3.1 WEED SPECIES COMPOSITION

Nineteen (19) weed species were recorded during the period of crop growth in both seasons. The weed species comprised of 10 broadleaf weeds, 7 grasses and 2 sedges (Table 2). The prevalence of both annual and perennial broadleaved weeds and grasses in this study may be a result of increased soil disturbance from previous tillage (Menallad et al., 2001). The weed species were generally more abundant in the early than in the late season. *Commelina benghalensis* L., *Gomphrena celosioides* Mart., *Boerhavia diffusa* L., *Talinum triagulare* (Jacq.) Willd., *Chromolaena odorata* (L.) R.M.King & H.Rob and *Digitaria horizontalis* Willd. which had high infestation in the early season were found with moderate infestation in the late season. This was possibly because the rainfall was generally more abundant and evenly distributed in the early season compared with the late season. It has been reported that rainfall affects weed species distribution and their competitiveness within a crop community (Shaidul et al., 2011).

3.2 EFFECT OF WEED MANAGEMENT METHODS ON WEED COVER SCORE AND BIOMASS

Butachlor and propaben and hoe-weeding treatments resulted in significant reduction in weed cover score and biomass compared with the weed check in early and late season (Table 3). Sole application of butachlor and propaben each at 2 kg a.i ha^{-1} reduced weed cover score similar to two hoe-weedings in both seasons.

Table 2: Weed species and their level of infestation in the early and late wet seasons of 2017

Weed species	Plant family	Level of infestation[a]	
Broad leaf weeds		Early	Late
Tridax procumbens Linn.	Asteraceae	***	***
Euphorbia heterophylla Linn.	Euphorbiaceae	***	***
Commelina benghalensis Burn.	Commelinaceae	***	**
Gomphrena celosioides Mart.	Amaranthaceae	***	**
Spigelia anthelmia Linn.	Loganiaceae	***	***
Boerhavia diffusa Linn.	Nyctaginaceae	***	**
Chromolaena odorata (L.) R. M. King and Robinson	Asteraceae	***	**
Talinum triagulare (Jacq.) Willd.	Portulacacae	***	**
Laportea aestuans (Linn.) Chew.	Urticaceae	**	**
Ipomoea triloba Linn.	Convolvulaceae	**	**
Grasses			
Digitaria horizontalis Willd.	Poaceae	***	**
Panicum maximum Jacq.	Poaceae	***	***
Axonopus compressors (Sw.) P. Beauv.	Poaceae	***	***
Eleusine indica Gaertn.	Poaceae	**	**
Rottboellia cochinchinensis (Lour.) Clayton	Poaceae	*	*
Cynodon dactylon (Linn) Pers.	Poaceae	***	***
Paspalum scrobiculatum Linn.	Poaceae	**	**
Sedge			
Cyperus rotundus Linn.	Cyperaceae	**	**
Cyperus esculentus Linn.	Cyperaceae	**	**

[a] Level of weed infestation was based on weed ground cover: *** - Highly infested (60-90 %), **- Moderately infested (30-59 %), *-Low infestation (1-29 %).

Table 3: Effect of weed management methods on weed cover score, dry weed biomass and groundnut fresh pod yield

	Weed cover score[a]		Weed biomass (g m^{-2})		Groundnut pod yield (kg ha^{-1})	
	Early season	Late season	Early season	Late season	Early season	Late season
Butachlor	6.6	7.2	153.0	178.2	722.9	524.2
Butachlor + shw[b] at 6 WAS[c]	3.2	3.6	100.9	70.7	1192.3	1136.7
Propaben	6.1	7.1	146.9	162.2	561.8	405.8
Propaben + shw[b] at 6 WAS	4.4	4.7	131.1	93.8	1217.3	1146.3
Two hoe-weedings	6.4	6.7	110.4	97.1	1144.6	1089.4
Three hoe-weedings	2.7	3.0	119.4	71.3	1285.7	998.8
Weedy check	8.5	8.1	206.1	230.1	402.1	287.8
LSD (5 %)	0.93	0.845	22.6	20.4	336.2	332.8

[a]Weed cover score was based on a scale of 0-10: where 0 represents no weed and 10 represents complete weed cover, [b]Shw – supplementary hoe weeding; [c]WAS – weeks after sowing.

This may be as a result of the efficacy of the herbicides in inhibiting weed root elongation, protein synthesis and other processes of blocking of weed development (Daramola et al., 2020). Butachlor and propaben at 2 kg a.i ha^{-1} each fb shw at 6 WAS reduced weed cover score similar to three hoe-weeding and better than two hoe-weeding or sole application of either herbicide in both seasons. Similarly, butachlor and propaben each followed by supplementary hoe-weeding resulted in significant reduction in weed biomass similar to two and three hoe-weeding and better than sole application of either herbicide in both early and late wet seasons (Table 3). This showed that sole herbicide application could not provide complete weed control without supplementary hoe weeding. The herbicides provided initial management of germinating weed seedlings but lost efficacy thereafter, thus allowing weed resurgence. The lowest weed biomass (100.9 and 70.7 g m^2 in the early and late seasons, respectively) was recorded in plots treated with butachlor fb shw at 6 WAS. The efficacy of this treatment may be attributed to the suppression of weeds by the herbicide at the start of groundnut growth and the removal of weeds by the supplementary hoe-weeding later in the growing season, both of which helped to control weeds before setting seed and gave the crop a competitive advantage over weeds coming in the second flush. These results have corroborated the results from report of Daramola et al. (2020) that integration of herbicide application and hoe weeding is superior to sole herbicide or sole manual hoe weeding.

3.3 EFFECT OF WEED MANAGEMENT METHODS ON GROUNDNUT YIELD

Regardless of the weed management method used, groundnut pod yield was higher in the early season than in the late season (Table 3). This was possibly because the rainfall was more abundant and evenly distributed in the former than in the later part of the season. All the weed management methods resulted in significantly higher groundnut pod yield than the weedy check in both seasons (Table 3). This result is in agreement with that of Ojelade et al. (2018) who reported increased yield of groundnut due to various weed control treatments owing to the increased availability of nutrient, light and space. Although manual hoe-weeding or sole herbicide application is presently the most common weed management method in groundnut production in Nigeria, the result of this study, showed that pre-emergence application of butachlor and propaben each followed by supplementary hoeing at 6 WAS resulted in significant increase in groundnut pod yield similar to two and three hoe-weedings and higher than sole herbicide application in both early and late cropping seasons (Table 3). This was probably because sole application of propaben or butachlor provided weed management only at the time of weed germination and shortly after emergence, but not at later stages of crop growth, when broad-leaved weeds with relatively late emergence pattern start to emerge. These results have corroborated the report of Mishra et al. (2017) that no single method, whether manual or chemical can provide the desired level of weed control efficiency under all situation.

In this study, the highest groundnut pod yield (1285.7 kg ha^{-1}) in the early season was recorded with three hoe-weedings. However, in the late season, the highest groundnut pod yield (1146.3 kg ha^{-1}) was recorded with pre-emergence application of propaben plus hoe weeding. Optimum groundnut pod yield recorded with three hoe-weedings and pre-emergence herbicides plus hoe-weeding may be attributed to their efficiency in reducing weed growth throughout the prolonged period of

Table 4: Total variable cost, revenue, gross margin and cost-benefit ratio of groundnut as affected by different weed management methods in early and late cropping seasons of 2017

	Early season				Late season			
	Total variable cost	Revenue[b]	Gross margin[c]	Cost-benefit ratio	Total variable cost	Revenue[b]	Gross margin[c]	Cost-benefit ratio
Treatments	(US\$ ha^{-1})[a]			(US\$ ha^{-1})[a]				
Propaben	333.3	797.6	464.3	1.4	312.4	578.4	266.0	0.9
Propaben + shw[d]	416.3	1315.5	899.2	2.2	374.4	1250.3	875.9	2.3
Butachlor	337.9	619.8	281.9	0.8	317.0	447.7	130.7	0.4
Butachlor + shw[d]	420.9	1343.1	922.2	2.2	379.0	1264.8	885.8	2.3
Hw[e] twice	457.9	1262.9	805.0	1.8	395.0	1202.0	807.0	2.0
Hw[e] thrice	540.9	1414.3	873.4	1.6	457.0	1102.1	645.1	1.4
Weedy check	292.9	443.6	150.7	0.5	272.6	317.5	44.9	0.2

Values are presented in US dollar ha^{-1}, [a]Nigeria Naira was converted to US dollars at exchange rate of 362.53 Naira to US\$1.00, [b]revenue was obtained by multiplying the groundnut pod yield by the steady market price (US\$ 1.1 kg^{-1}), [c]gross margin = revenue from groundnut yield output minus variable cost of production [d]shw – supplementary hoe weeding, [e]Hw – hoe-weeding

crop growth, which probably led to increased supply and use of growth resources (Khaliq et al., 2012). Unchecked weed growth throughout the period of crop growth reduced groundnut pod yield by 72.9 and 74.8 % in the early and late seasons, respectively (Table 3).

3.4 EFFECT OF WEED MANAGEMENT METHODS ON COST OF PRODUCTION AND PROFITABILITY OF GROUNDNUT

The weed management methods incurred higher cost of production than the weedy check (Table 4). This showed that the cost of weed management takes bulk of the total production cost as earlier reported by Adigun and Lagoke (2003). Regardless of the weed management methods, the total cost of production was generally lower in the late compared to the early cropping season (Table 3). This may be due to the reduction in cost of weed management occasioned by the reduced weed growth in the former than in the latter. This result have corroborated that of Adigun and Lagoke (2003) who reported that crop production is more cost effective in the early season than in the late season due to reduction in weed growth in the former than in later. Butachlor and propaben each applied alone at 2 kg a.i ha^{-1} or followed by hoe-weeding incurred lower cost of production than two and three hoe-weedings in both seasons (Table 4). This may be attributed to the reduction in labor requirement for herbicide application compared with the labor required for multiple hoe-weedings. Overfield et al. (2001) have earlier reported that herbicide application required 1.3 person-days ha^{-1} while manual hoe weeding required 39.2 person-days ha^{-1}. The result of this study is also in agreement with the report of Gouse et al. (2006) that weed removal through herbicide application required only 2 hours of labor per hectare, whereas manual hoe-weeding required 250 hours. Of all the weed management methods, three hoe-weeding incurred the highest cost of production (\$540.0 and \$457.0 ha^{-1} in early and late seasons, respectively) followed by two hoe-weedings (\$457.9 and \$395.0 ha^{-1} in early and late seasons, respectively. Higher cost of production incurred with hoe-weedings compared with herbicidal treatment may be attributed to the accumulated cost of manual hoe-weeding which is usually expensive. This result is in agreement with that of by Adigun and Lagoke (2003), who reported that hoe weeding is expensive and causes a lot of drudgery.

There was higher total revenue and gross margin in the early season than in the late season (Table 4). This was probably as a result of higher yield occasioned by better rainfall in the former than in the latter. Butachlor and propaben at 2 kg a.i ha^{-1} each followed by hoe weeding gave higher total revenue and gross margin than two hoe-weeding or sole application of either herbicide in both early and late season. These treatments also gave higher total revenue, gross margin and cost-benefit ratio than three hoe weeding in the late season. Highest total revenue ($1414.3) in the early season was recorded with three hoe weedings while the highest total revenue ($1264.8) in the late season was recorded with butachlor plus hoe weeding. Although three hoe-weedings gave the highest revenue in the early season, the gross margin and cost-benefit ratio obtained was lower than those of propaben and butachlor each followed by supplementary hoe eeding. Similarly, three hoe weeding gave lower gross margin and cost-benefit ratio than two hoe-weeding and propaben or butachlor followed by supplementary hoe-weeding in the late season. This showed that the gain in revenue from three hoe-weedings compared to herbicide plus hoe-weeding treatments was nullified by accumulated labour cost for hoe weeding. Application of butachlor and propaben plus hoe weeding gave higher gross margin and cost-benefit ratio than three hoe-weeding treatments in both seasons. The highest gross margin ($922.2 and $885.8 in the early and late seasons, respectively) was recorded with butachlor at 2 kg a.i ha^{-1} plus hoe weeding. The highest cost-benefit ratio (2.2 and 2.3 in the early and late seasons, respectively) was recorded with propaben and butachlor each followed by hoe-weeding (Table 4). This showed that pre-emergence herbicides plus hoe-weeding provided high yield at relatively lower cost. This result have corroborated previous report of Khaliq et al. (2012) that weed control with herbicides provided higher gross margin and cost-benefit than manual weeding. Lowest revenue, gross margin and cost-benefit ratio (0.5 and 0.2 in the early and late seasons, respectively) was recorded in the weedy plot (Table 4).

4 CONCLUSION

This study demonstrated the effectiveness of integrated weed management for efficient weed management, higher pod yield, revenue, gross margin and cost-benefit ratio than sole herbicide application or manual hoe weeding in groundnut production in both early and late seasons. Farmers can therefore reduce labour cost for manual hoe weeding with pre-emergence application of butachlor or propaben. This can be supplemented by one hoe weeding at 6 weeks after sowing for season-long weed management, optimum groundnut pod yield and ultimately higher profit in groundnut production.

5 REFERENCES

Adigun, J. A., Lagoke, S. T. O. (2003). Weed control in transplanted rain and irrigated tomatoes in the Nigerian savanna. *Nigerian Journal of Weed Science*, *16,* 23-29.

Adigun, J. A., Adeyemi, O. R., Lagoke, S. T. O., Olorunmaiye, P M., Daramola, O. S., Babatunde, O. A. (2016). Influence of inter-row spacing and weed control in groundnut (*Arachis hypogeae* L.) *Journal of Agricultural Science and Environment, 16*(1), 22-35.

Adigun, J. A., Adeyemi, O. R., Daramola, O. S., Odueme, P. U., Fadeyi, O. J. (2017). Growth and yield performance of groundnut (*Arachis hypogeae* L.) as affected by row spacing and weed control methods in the Nigerian forest–savannah transition zone. *Nigerian Journal of Ecology, 16*(1), 22-35.

Adigun, J. A., Daramola, O. S., Adeyemi, O. R., Ogungbesan A. (2018). Impact of nitrogen levels and weed control methods on growth and yield of okra (*Abelmoschus esculentus* (L.) Moench) in the Nigerian forest-savanna. *Journal of Experimental Agriculture International, 20*(2), 1-11. https://doi.org/10.9734/JEAI/2018/39107

Bado, B. V., Lompo, F., Bationo, A. (2012). Nitrogen recoveries and yields improvement in cowpea-sorghum and fallow-sorghum rotations in West Africa Savannah. *Journal of Agricultural Science and Technology,* 2758–2767.

Bhagirath, S. C., Chauhan, J. H., Opeña, L. (2013). Effect of plant spacing on growth and grain yield of soybean. *American Journal of Plant Sciences*, *4,* 2011-2014. https://doi.org/10.4236/ajps.2013.410251

Birthal, P. S., Nigam, S. N., Narayanan, A. V., Kareem, K. A. (2011). An economic assessment of the potential benefits of breeding for drought tolerance in crops: A case of groundnut in India. *Research Bulletin no. 25.* Patancheru: International Crops Research Institute for the Semi-Arid Tropics.

Chauhan, B. S., Singh, R. G., Mahajan, G. (2012). Ecology and management of weeds under conservation agriculture: A review. *Crop Protection, 38*, 57-65. https://doi.org/10.1016/j.cropro.2012.03.010

Daramola, O. S., Adeyemi, O. R., Adigun J. A., Adejuyigbe, C. O. (2019). Crop-weed association and inter-relationships in soybean-weed ecosystem in the transition zone of south-west Nigeria. *Nigerian Journal of Ecology 17*(2), 97-114.

Daramola, O. S., Adeyemi, O. R., Adigun J. A., Adejuyigbe, C. O. (2020). Weed interference and control as affected by row spacing in the transition zone of south-west Nigeria. *Journal of crop Improvement, 34*(1), 103-121. https://doi.org/10.1080/15427528.2019.1674759

Datta, A., Ullah, H., Tursun, N., Pornprom, T., Knezevic, S. Z., Chauhan, B. S. (2017). Managing weeds using crop competition in soybean (*Glycine max* (L.) Merr.) *Crop protection, 95*, 60-68. https://doi.org/10.1016/j.cropro.2016.09.005

Food and Agricultural Organizations of the United Nations (FAOSATAT). *Statistical Data Base*; Food and Agricultural Organizations of the United Nations: Rome, Italy, 2018.

Gouse, M., Jenifer, P., Colin, T. (2006). Output and labour effects of GM maize and minimum tillage in a communal area of Kwazulu-Natal. *Journal of Development Perspectives, 2*(2), 71-86. https://doi.org/10.35648/20.500.12413/11781/ii259

Imoloame, E. O. (2014). The effects of different weed control methods on weed infestation, growth and yield of soybeans (*Glycine max* (L) Merrill) in the southern guinea savanna of Nigeria. *Agro search, 14*(2), 129-143. https://doi.org/10.4314/agrosh.v14i2.4

Janila, P., Nigam, S. N., Manish K., Pandey, P., Nagesh, R., Varshney, K. (2013). Groundnut improvement: use of genetic and genomic tools. *Frontier in Plant Science*, *4*, 23 https://doi.org/10.3389/fpls.2013.00023.

Kercher, S. M., Frieswyk C. B., Zedler, J. B (2003). Effects of sampling teams and estimation methods on the assessment of plant cover. *Journal of Vegetable Science, 14,* 899-906. https://doi.org/10.1111/j.1654-1103.2003.tb02223.x

Khaliq, A., Matloob. A., Ahmed, N., Rasul, F., Awan, I. U. (2012). Late POST emergence chemical weed control in direct seeded fine rice. *Journal of Animal and Plant Science, 22,* 1101-1106.

Maurice, D. C., Amaza, P. S., Tella. M. O. (2005). Profitability of rice based cropping patterns among dry season Fadama Farmers in Adamawa State, Nigeria. *Applied and Tropical Agriculture, 10,* 13-16.

Menalled, F. D., Gross, K. L., Hammond, M. (2001). Weed aboveground and seed bank community responses to agricultural management systems. *Ecology Application, 11*, 1586–1601. https://doi.org/10.1890/1051-0761(2001)011[1586:WAASCR]2.0.CO;2

Mishra, J. S., Rao, A. N., Singh, V. P., Kumar, R. (2017). Weed management in major field crops. *Advances in Weed Management, 4,* 1-21.

Nikoa, R, M., Owen, D. K., Swanton. C. J. (2015). Weed Abundance, Distribution, Diversity, and Community Analyses. *Weed Science, 63,* 64-90. https://doi.org/10.1614/WS-D-13-00075.1

Ojelade, O. B., Lagoke, S. T. O., Adigun, J. A., Babalola, O. A., Daramola, O. S., Osipitan, O. A. (2018). Intra-row spacing and weed control influence on growth and yield of groundnut (*Arachis hypogea* L.). *Journal of Agricultural Science, 6*(4), 1-11.

Overfield, D, Murithi, M. F., Muthamia, J. N., Ouma, J. O., Birungi, K. F., Maina, J. M…Terry, P. T. (2001). Analysis of the constraints to adoption of herbicides by smallholder maize growers in Kenya and Uganda. *Proceedings of the BCPC Conference Weeds 2001, 907-912*. Lagos, Nigeria.

Pannell, D. J., Marshall, G. R., Barr, N., Curtis, A., Vanclay, F., Wilkinson, R. (2006). Understanding and promoting adoption of conservation practices by rural landholders. Australian *Journal of Experimental Agriculture, 46*(11), 1407-1424. https://doi.org/10.1071/EA05037

Sepat, S., Thierfelder, C., Sharma, A. R., Pavuluri, K., Kumar, D., Iquebal, M. A., Verma, A. (2017). Effects of weed control strategy on weed dynamics, soybean productivity and profitability under conservation agriculture. *Field Crops Research, 210,* 61-70. https://doi.org/10.1016/j.fcr.2017.05.017

Shadiul, H. B., Min, Y. E, Romij, M. D, Tea, S. P, Hang, W. K, Do, S. K, Park, K. W. (2011). Weed population dynamics under climatic change. *Asian journal of Turf Grass Science, 25(1),* 174-182.

Sogut, T., Ozturk, F., Kizil, S.(2016). Effect of sowing time on peanut (*Arachis hypogea* L.) cultivars: II. Fatty acid composition. *Agriculture and Agricultural Science Procedia*, *10*(1), 76-82. https://doi.org/10.1016/j.aaspro.2016.09.018

Tunku, P., Lagoke, S. T. O., Ishaya, D. B. (2007). Evaluation of herbicides for weed control in irrigated garlic at Samaru, Nigeria. *Crop Protection, 26,* 642-646. https://doi.org/10.1016/j.cropro.2006.05.017

doi:10.14720/aas.2020.116.1.1719 Original research article / izvirni znanstveni članek

Impact of dust accumulation on yield and yield components of soybean

Sharife HABIBPOUR [1], Majid AMINI DEHAGHI [1, 2], Mohammad Eghbal GHOBADI [3], Alaeddin KORDENAEEJ [1]

Received June 08,2020; accepted September 02, 2020.
Delo je prispelo 08. junij 2020, sprejeto 02. september 2020.

Impact of dust accumulation on yield and yield components of soybean

Abstract: This study aimed to characterize if dust sprayed on soybean foliage impacts its yield and yield component characteristics. In 2017 and 2018, soybean [*Glycine max* (L.) Merr.] was planted using a factorial randomized complete block design with three replicates. Plants were sprayed with a 20 g m^{-2} of dust at four stages of the growth cycle, including third-node, the beginning of flowering, the beginning of podding, and the beginning of seed formation. Dust spraying was then continued twice weekly until the late full seed stage. Plant measurements included yield, yield components, stomatal conductance, peroxidase, and superoxide dismutase antioxidant enzymes activities. Results showed that depending on the time of application, the dust coverage created a range of yield loss in soybeans, most likely due to a reduction in stomatal conductance, grains plant^{-1} and 100-seed mass. Therefore, soybean fields that are regularly exposed to dust might be subjected to reduced yield.

Key words: peroxidase; superoxide dismutase; stomatal conductance

Vpliv nalaganja prahu na pridelek in njegove komponente pri soji

Izvleček: Namen raziskave je bil ugotoviti, če nalaganje prahu na listje soje vpliva na njen pridelek in njegove komponente. V letih 2017 in 2018 je bila posejana soja [*Glycine max* (L.) Merr.] v popolnem faktorskem poskusu s tremi ponovitvami. Rastline so bile posipane z 20 g m^{-2} prahu v štirih razvojnih fazah, ob pojavu tretjega nodija, v začetku cvetenja, v začetku razvoja strokov, in v začetku tvorbe semen. Prašenje je potekalo dvakrat tedensko do dokončnega razvoja semen. Meritve na rastlinah so obsegale meritev pridelka in njegovih komponent, prevodnost rež, in meritve aktivnosti antioksidacijskih encimov peroksidaze in superoksid dismutase. Rezultati so pokazali, da je odvisno od časa nanosa prah zmanjšal pridelek soje, najverjetneje zaradi zmanjšanja prevodnosti rež, zmanjšanja števila zrna na rastlino in mase stotih semen. Zaključimo lahko, da se zmanjša pridelek soje na poljih, ki so redno izpostavljena prašenju.

Ključne besede: peroksidaza; superoksid dismutaza; prevodnost rež

---

[1] Shahed University, Department of Agronomy, Tehran, Iran
[2] Corresponding author, e-mail: amini@shahed.ac.ir
[3] Razi University, Department of Agronomy and Crop Science, Kermanshah, Iran

1 INTRODUCTION

Natural factors and human activities lead to the production of dust particles. In Iran, the primary sources of dust storms, which influence the western and central regions, originate mainly from the deserts of Iraq and Saudi Arabia (Pirsaheb et al., 2014). In July 2009, the dust had adverse effects on Iran's agricultural lands and industrial areas (Hojati et al., 2012). In Yazd city, located in the deserts of Iran, the average airborne dust particles were more than 200 µg m^{-3} over five months, leading to a significant loss (between 3 % to 30 %) in crops yield (Shahsavani et al., 2011). According to the meteorological organization statistics of Iran's Kermanshah province, dusty days occur mainly in spring and summer seasons (Doabi et al., 2017). Due to the dynamic characteristics of dust, particles with a diameter of fewer than ten µm can be transported by the wind for several thousands of kilometers (Zia-Khan et al., 2015).

Undoubtedly, dust causes many environmental impacts, such as loss of soil fertility or direct damages to crops, resulting in reduced agricultural products and, thus, large-scale economic losses (Walia et al., 2019). Besides, there is no regular natural removal of dust particles on the plant leaves by strong winds and rain, as rainfall is scarce, and the wind brings more dust rather than alleviating the situation (Zia-Khan et al., 2015). Dust-covered leaves provide less light for photosynthesis. Also, dust reduces the conductivity of the leaf stomata leading to decreased plant's biomass and yield. Zia-Khan et al. (2015) believe that dust accumulation on leaf surfaces induces water stress-like conditions.

Investigations on dust have mainly focused on the impacts of the different kinds of dust on morpho-physiological changes in plants (Drack & Vázquez, 2018; Hatami et al., 2018; Siqueira-Silva et al., 2017; Siqueira-Silva et al., 2016). Stone crusher dust led to a decrease in grain yield of rice (*Oryza sativa*. L.) (Sharma & Kumar, 2016) and gram (*Cicer arietinum* L.) (Sharma & Kumar, 2015). Also, cement dust reduced yield, and one thousand-seed mass in wheat (Chaurasia et al., 2014; Hatami et al., 2018). A significant reduction in stomatal conductance has been reported under the influence of dust (Hirano et al., 1995; Zia-Khan et al., 2015). The smaller the particle size, the higher the effect of dust in reducing stomatal conductance so that fine particles less than five µm in diameter can interfere with the mechanism and function of the stomata (Singh et al., 2018).

Soybean (*Glycine max* L.) is one of the most important crops with many applications in food products, animal feed, and industries (Gnoinsky et al., 2019). It also has unique nutritional properties such as high content of proteins, oil, fiber, vitamins. Therefore the worldwide demand for soybean is at a high level (Lokuruka, 2011). Even with advances in farming practices, crop yields are still strongly linked to climate change (Glotter & Elliott, 2016). Therefore, changes in field conditions, such as dust deposition, might affect crop performance and physiological properties.

Despite its strategic role, no study has been conducted on the impact of dust on soybean at different stages of growth under field conditions. Therefore, the objective of this study was to determine if the dust used at different growth stages influence soybean yield and yield components. Besides, stomatal conductance and the activities of peroxidase and superoxide dismutase antioxidant enzymes were assayed.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL DESIGN

The study was conducted in Kermanshah, Iran (34° 31′ N, 47° 09′ E, 1319 m above the sea level) during two consecutive years (2017-2018). The experiment was laid out in the form of a factorial randomized complete block design with three replicates (Fig. 1).

In each year, the experimental farm was divided into 24 plots (8 plots in each replicate). Each experimental plot consisted of 4 planting lines 4 m long and 50 cm apart. A distance of 0.5 m was). Soybean seeds (*Glycine max* 'Hobbit'), inoculated with *Rhizobium japonicum* Buchanan 1926 considered between the experimental plots, and 1.5 m between the experimental blocks (replicates was planted at a depth of 5 cm on rows 8 cm apart). The plants were watered by the surface irrigation method.

2.2 DUST TREATMENT

During the two years of the experiment, the effect of dust deposition on soybeans was investigated at four different time intervals (Fig. 2).

The control plants were rinsed with a hand sprinkler. Rinsing was done twice weekly to increase accuracy and ensure the absence of dust on control plants. Control plants were also rinsed by each occurrence of a natural dust storm. The water used for rinsing the control treatment was added to the soil of other plots to prevent possible errors. Dust particles had a natural origin and were collected using a gravimetric method

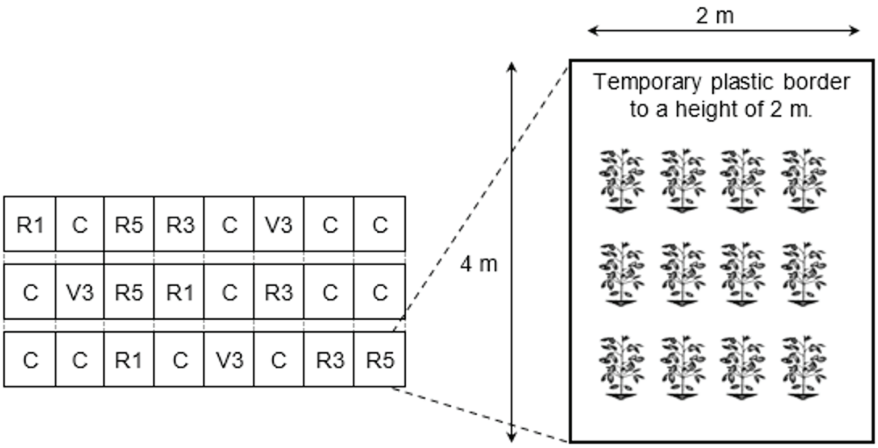


Figure 1: Schematic of the experimental design showing the treatments and the layout of the wind-proof spray chamber (zoom out). In each year of the experiment, the random distribution of treatments within the plots was different. The plants were exposed to dust from V3 (the third-node stage), R1 (the beginning of flowering), R3 (the beginning of podding), and R5 (the beginning seed formation); and C defines the control plots.

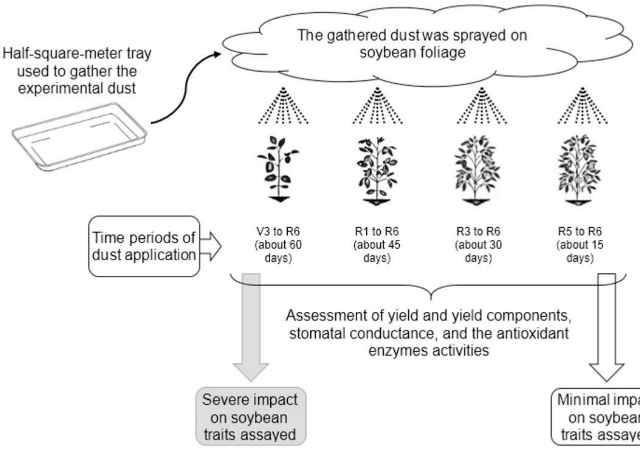


Figure 2: Schematic of whole of the experiment showing the dust application on soybeans at four different growth stages (time intervals).

from soybean cultivating sites exposed to dust storms for one year. For this purpose, half-square-meter trays were used to gather the dust deposited during dust storms. Chemical analysis of the experimental dust was performed by Doabi et al. (2017) (Table 1).

Table 1: Results of the chemical analysis (mg kg−1) of the experimental dust samples (Doabi et al., 2017)

Element	Minimum	Maximum	Mean
Zn	132	700	238.29
Cu	24	256	46.67
Ni	60	245	123.72
Cr	44	147	73.6
Mn	400	695	495.16
Fe	20,750	35,562	28,703.94

2.3 DUST APPLICATION

In each year, dust spraying was done at four different times based on the plant growth stages, including V3 (third-node), R1 (the beginning of flowering), R3 (the beginning of podding), and R5 (the beginning of seed formation). Spraying was then continued until the late R6 (full seed) stage twice weekly (Fig. 2). At each event, a 20 g m^{-2} of dust was sprayed with a roughly uniform layer using a manual dust-generator. The machine was firstly calibrated based on the amount of dust accumulated on

Table 2: Meteorological data during the two years of the experiment extracted from the meteorological station at the Razi Faculty of Agriculture, Kermanshah, Iran

	Jun		Jul		Aug		Sep		Oct		Nov	
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
RH (%)	17.27	24.49	12.16	13.23	10.88	14.86	11.74	15.62	21.59	42.67	50.44	70.75
Max T (°C)	34.86	34.66	39.1	40.21	38.76	39.89	35.33	34.91	26.52	25.79	17.65	15.14
Min T (°C)	13.52	13.55	18.26	19.07	18.75	16.89	13.15	13.24	7.95	10.59	3.81	5.65
Mean T (°C)	25.67	25.31	30.02	30.87	29.76	29.4	25.16	24.61	17.27	17.84	10.1	9.77
AP (mm)	0	2.2	0	0	0	0	0	0.21	0.2	71.46	39.73	126.33
DD	2	9	5	4	0	3	2	1	7	4	5	0
AWS (m s^{-1})	3.14	2.94	3	3.36	3.12	2.95	2.68	2.55	2.83	2.61	2.33	2.74

RH: the relative humidity, *T*: Temperature, *AP*: Average Precipitation, *DD*: The number of dusty days, *AWS*: Average wind speed

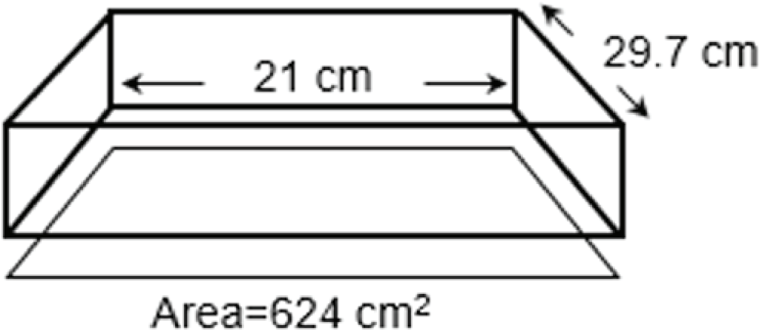


Figure 3: The sampling container used to calibrate the dust generator and to quantify the amount of dust deposited on plant leaves.

the surface of an A4 paper (624 cm², Fig. 3). In addition, to determine the amount of dust deposited on plant leaves in each treatment, a rectangular cube glass container with a size of 27 × 29.7 cm was placed inside each plot. After each treatment, the amount of dust deposited on the paper surface inside the container was measured (Fig. 3).

A wind-proof spray chamber was used to reduce wind disturbances during application and to ensure that dust was applied evenly to the treatment area, and also to avoid interference between the dust treatments (Fig. 1). The dust was sprayed when the air was constant without wind. After each rainfall, dust treatment was re-applied to soybean plants. Weather data from the weather station at the Razi Faculty of Agriculture (the nearest weather station) was recorded (Table 2).

2.4 MEASUREMENTS

In each year, yield and yield component characters were measured in the late full maturity (R8) stage when 95 % of the pods have reached their full mature color. Antioxidant enzymes were measured in the late R6 stage when the plants had the maximum leaf area and height.

2.4.1 Antioxidant enzymes activity

The activity of the peroxidase enzyme (POD) was measured, according to MacAdam et al. (1992), with minor modifications. The reaction mixture contained 50 µg guaiacol and 50 µl of 3 % H_2O_2 in 0.1 mM phosphate buffer. The reaction was started by adding 50 µl of extract. The reaction was started by adding 50 µl of extract. Then, the guaiacol peroxidase activity was determined spectrophotometrically by measuring the absorbance increase at 436 nm for 3 minutes at 15-second intervals.

The activity of the superoxide dismutase (SOD) was determined consistent with Beauchamp and Fridovich (1971) method. A 50 µl of the extract was dissolved in one ml of reaction buffer, including 50 mM potassium phosphate buffer (pH 7.8), 75 μM NBT, 13 ml L-methionine 0.1 mM, EDTA, and two mM riboflavin. The mixture was placed in a light chamber for 15 minutes, and then its optical absorption rate was read at nm560 wavelength.

2.4.2 Stomatal conductance

The stomatal conductance (SC, mmol m^{-2}) was measured between 9-12 am by a leaf porometer (SC-1 Decagon Devices, Inc., USA) over ten leaves randomly selected from each plot.

2.4.3 Yield and yield components

Seeds of the third and fourth planting lines of each plot were harvested, and then weighed based on 12 % moisture content (R8 stage), and converted to kg ha^{-1}. Besides, some of the most important soybean yield components were also measured, including Plant Height (PH), Seeds Pod^{-1} (SPO), Seeds $Plant^{-1}$ (SPL), Pods $Plant^{-1}$ (PPL), 100-Seed Mass (HSM), and Harvest Index (HI).

2.4.4 Statistical analysis

Data were analyzed using the PROC GLM procedure of SAS software V. 9.1 (SAS Institute, Cary, NC, USA). A pairwise comparison of means was performed using the t-test (LSD) method at the 0.05 level of probability. All data collected during the two growing seasons were evaluated in a combined analysis format and then analyzed for differences among treatments over the two years, and presented in the combined form not individually within each year.

3 RESULTS

Table 2 shows the metrological data. In 2017, there were 2, 5, and 0 days with dust in the January, July, and August, respectively, coincide with V3-R1 stages, while there were 2, 7, and 5 days with dust for September, October, and November, respectively, coincide with R5-R8 stages. Therefore, in 2017, there were more days with dust at the end of the growth phase. Conversely, there were more days with dust in the early months of the growing season than the final months for the second year of the experiment. Furthermore, the wind speed on the ground was similar during both years of the investigation. Due to higher rainfall, the relative humidity was relatively higher in the second year.

3.1 EFFECT ON YIELD AND YIELD COMPONENTS

Results showed that dust by stage interaction had significant effects on yield and yield-related traits assayed (Table 3 and Fig. 4). As shown in Table 4 and Figure 4, the responses of yield and yield-related traits to dust application had the same behavior. First, their values differed significantly between dust-treated and control plants, second, the lowest values were obtained when the dust was applied from the V3 stage, and third, after the control, the highest values were observed when the plants were exposed to dust from the R5 stage. On average, over the two years, soybean seed yield varied from 2040.84 kg ha^{-1} to 481.42 kg ha^{-1}. Also, on average, seed yield declined significantly by 73.00 %, 53.33 %, 42.73 %, and 34.47 % in plots dusted from V3, R1, R3, and R5 stages, respectively (Fig. 4). Similarly, the same reduction schemes were also found for PH, SPL, PPL, SPO, HSM, and HI (Table 4). The analysis of standardized regression showed that SPL and HSM with 0.66 and 0.35 had the most significant positive direct effect on seed yield, respectively, while PH and PPL with -0.13, and -0.10 had negative direct effects on seed yield (data not shown).

3.2 EFFECT ON ANTIOXIDANT ENZYMES ACTIVITY AND STOMATAL CONDUCTANCE

The activity of antioxidant enzymes differed significantly between dust-treated and control plants. As shown in Figures 5 and 6, the antioxidants responses to dust application had the same pattern in both POD and SOD. Both enzymes assayed showed a significant activity under dust treatment and peaked when the plants were exposed to dust from the V3 stage. However, the antioxidants showed less activity when spraying was done in the later stages of growth (Figs 5 and 6). POD activity was 32, 25, 12, and 6 times higher than the control when plants were exposed to dust form V3, R1, R3, and R5 stages, respectively.

Similarly, SOD activity increased by 88.08 %, 65.51 %, 49.31 %, and 29.28 % for those plants exposed to dust form V3, R1, R3, and R5 stages, respectively. Therefore, under the influence of dust, the activity of POD showed a further increase compared with SOD.

On average, over the two years, the stomatal conductance varied from 9.91 to 26.22 mol CO_2 m^{-2} s^{-1} (Table 4). Exposure to dust resulted in a significant loss in

Table 3: Combined analysis of variance of the effect of dust deposition on yield, yield component, and antioxidant enzymes of soybean (*Glycine max*) at different growth stages

		Mean squares									
Source	DF	SOD	POD	PH	SPL	PPL	SPO	HSM	HI	SY	SC
Year	1	0.0001 ns	0.00001 ns	25420 **	4.39 ns	1020.1 ns	8.8 ns	0.043 ns	1686.37 ns	188.42 ns	0.04 ns
Rep (Year)	4	0.33 **	0.00002 ns	43.88 *	173.45 **	1.7 **	1.27 **	2.07 **	1554.68 **	705343 **	2.55 **
Stage	3	0.39 **	0.01 **	48.19 **	78 ns	61.63 *	0.49 *	13.93 **	110.68 *	613985 *	98 **
Dust	1	8.38 **	0.08 *	244.98 ns	1362.35 ns	268.66 ns	4.57 ns	144.49 *	4137.95 ns	12138819 ns	1193.71 **
Year*Stage	3	0.0003 ns	0.00003 ns	0.07 ns	9.11 ns	5.83 ns	0.04 ns	0.0001 ns	4 ns	24184 ns	0.03 ns
Year*Dust	1	0.0001 ns	0.00004 ns	7.02 **	293.44 *	5.71 ns	0.24 ns	0.11 **	1402.81 **	740754 *	0.02 ns
Stage*Dust	3	0.39 **	0.01 **	48.19 **	78 *	61.63 *	0.49 *	13.93 **	110.68 *	613985 *	98 **
Year*Stage*Dust	3	0.0003 ns	0.00003 ns	0.07 ns	9.11 ns	5.83 **	0.04 ns	0.0001 ns	4 ns	24184 ns	0.03 ns
Error	28	0.05	0.0002	11.43	22.98	0.33	0.13	0.22	214.56	93571.96	0.37

SOD: superoxide dismutase activity; POD: peroxidase activity; PH: plant height; SPL: seeds per plant; PPL: pods per plant; SPO: seeds per pod; HSM: hundred seed mass; HI: harvest index; SY: seed yield; SC: Stomatal conductance; DF: degree of freedom; *: $p < 0.1$; **: $p < 0.05$; ns: not significant. The effect of the year has been considered as random.

Table 4: The effect of desert dust on yield and yield component of soybean (*Glycine max*) at different growth stages

Dust	stage	PH (cm)	SPL	PPL	SPO	HSM (g)	SC (mol CO_2 m^{-2} s^{-1})	HI (%)
Dust free (control)		53.37 ± 10.62 a	33.38 ± 3.47 a	25.96 ± 2.24 a	2.24 ± 0.35 a	12.22 ± 0.34 a	26.22 ± 0.82 a	2040.84 ± 219.76 a
Dust application	V3	44.53 ± 10.27 d	16.26 ± 1.28 d	15.97 ± 1.35 b	1.11 ± 0.1 c	9.19 ± 0.2 c	9.19 ± 0.2 e	9.85 ± 0.32 d
	R1	46.36 ± 10.09 c	21.41 ± 0.36 cd	18.91 ± 1.39 b	1.63 ± 0.21 b	14.51 ± 0.15 c	14.51 ± 0.15 d	17.03 ± 1.03 c
	R3	52.13 ± 10.14 b	25.18 ± 2.21 bc	24.76 ± 2.42 a	1.63 ± 0.17 b	18.81 ± 0.5 b	18.81 ± 0.5 c	20.9 ± 0.29 b
	R5	52.4 ± 10.12 b	28.06 ± 1.27 ab	25.25 ± 2.48 a	2.1 ± 0.19 a	22.46 ± 0.62 b	22.46 ± 0.62 b	23.91 ± 0.39 b
LSD		0.4752	5.5452	4.4382	0.3739	0.0189	0.2994	3.6735

PH: plant height; SPL: seeds $plant^{-1}$; PPL: pods $plant^{-1}$; SPO: seeds pod^{-1}; HSM: one hundred seed mass; SC: Stomatal conductance; HI: harvest index

SC compared to the control plants (Table 4). Besides, the highest considerable decline in SC was found for plots treated with dust from the V3 stage (about 64.95 % decrease, compared with control).

At each of the growth stages, the dust was sprayed weekly from V3 (the third-node stage), R1 (the beginning of flowering), R3 (the beginning of podding), R5 (the beginning of seed formation), until the late full seed stage (R6).

In each column, means that do not share a letter are significantly different according to the LSD test (α = 0.05).

Compared with the control, the application of dust from the R1, R3, and R5 stages resulted in 44.64 %, 28.24%, and 14.34% decrease in stomatal conductance, respectively. Therefore, SC was significantly higher for plots dusted from the R5 stage. The study of the Pearson correlation coefficients showed that SY was positively correlated with SPL, PPL, SPO, HSM, HI, and SC. In contrast, the correlations between SY with POD and SOD were significantly negative (Table 5).

The antioxidant enzymes were negatively correlated with yield and yield components. Conversely, yield and yield components were positively correlated with stomatal conductance. Pods plant^{-1} was negatively correlated with PH. Principal components analysis revealed that in 2017, HI, SY, and HSM had the highest load on the first principal component, respectively. Similarly, in 2018, HSM, PP, and SY had also the highest load on PC1, respectively. Considering that the PC1 justifies the most

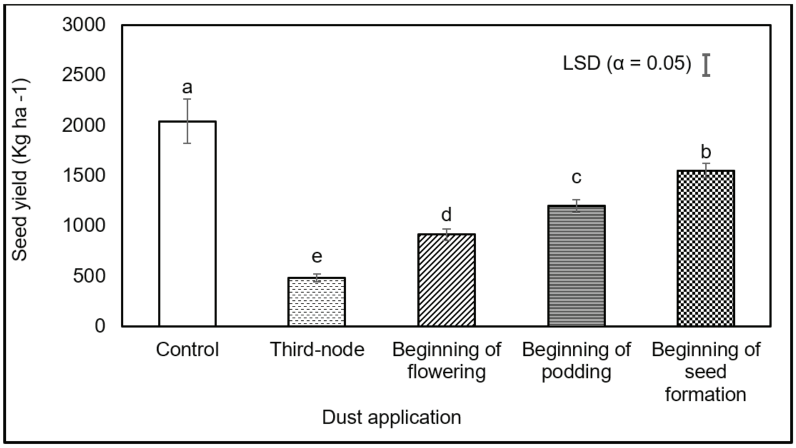


Figure 4: The effect of desert dust deposition on soybean seed yield. The dust was sprayed weekly on soybean leaves from each of the four different growth stages shown in the figure to the late full seed stage. The letters shown at the top of the columns indicate significant differences based on the LSD method (α = 0.05)

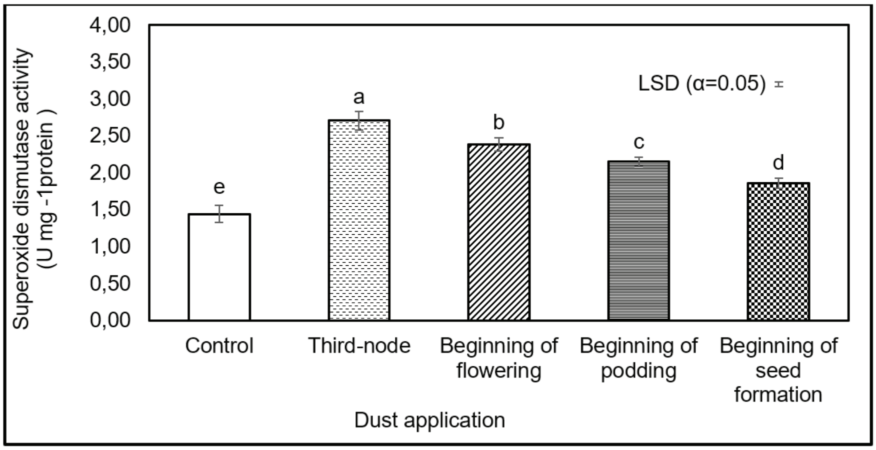


Figure 5: The effect of desert dust deposition on superoxide dismutase activity in soybean leaves. The dust was sprayed weekly on soybean leaves from each of the four different growth stages shown in the figure to the late full seed stage. The letters shown at the top of the columns indicate significant differences based on the LSD method (α = 0.05)

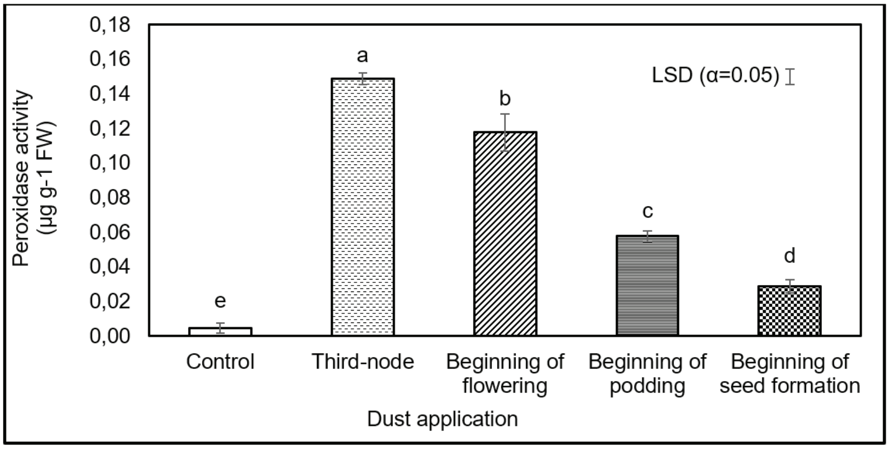


Figure 6: The average peroxidase activity in soybean leaves under the impact of desert dust deposition. The dust was sprayed weekly on soybean leaves from each of the growth stages shown in the figure to the full seed stage. The letters shown at the top of the columns indicate significant differences based on the LSD method (α = 0.05)

Table 5: Pairwise Pearson correlations between the traits studied on soybean (Glycine max L.) under the impact of dust deposition at different growth stages

	SOD	POX	PH	SPL	PPL	SPO	HSW	HI	SY
POX	0.87**	1							
PH	-0.11	-0.12	1						
SPL	-0.44**	-0.72**	0.1	1					
PPL	-0.49**	-0.59**	-0.66**	0.378*	1				
SPO	-0.62**	-0.53**	0.69**	0.15	-0.14	1			
HSW	-0.89**	-0.95**	0.15	0.7**	0.55**	0.62**	1		
HI	-0.22	-0.48**	-0.27	0.86**	0.49**	-0.21	0.48**	1	
SY	-0.62**	-0.84**	0.09	0.97**	0.46**	0.315*	0.84**	0.82**	1
SC	-0.86**	-0.97**	0.14	0.72**	0.58**	0.51**	0.96**	0.47**	0.83**

* and ** indicate that correlation is significant at the 0.05, 0.01 level, respectively.

SOD: superoxide dismutase activity, POX: peroxidase activity, PH: plant height; SPL: seeds plant^{-1}; PPL: pods plant^{-1}; SPO: seeds pod-1; HSM: one hundred seed mass; HI: harvest index; SY: seed yield, SC: Stomatal conductance.

variance therefore, HSM and SY experienced the highest variations under the influence of dust accumulation in both years (Fig. 7).

SOD: superoxide dismutase activity; POD: peroxidase activity; PH: plant height; SPL: seeds per plant; PPL: pods per plant; SPO: seeds per pod; HSM: hundred seed weight; HI: harvest index; SY: seed yield; SC: Stomatal conductance.

4 DISCUSSION

In this study, dust resulted in a significant decrease in yield and yield components of soybean compared with the control. Previous studies have shown that dust pollution resulted in a yield loss in cotton (Abdullaev & Sokolik, 2020), potato (Tomar et al., 2018), black gram (*Phaseolus mungo* L.) (Babu et al., 2018), rice (Sett, 2017; Sharma & Kumar, 2016), and grapevine (Karami et al., 2017). Hatami et al. (2018) studied the effects of desert dust on yield and yield components of cowpea and found that exposure to desert dust significantly decreased biological yield and seed yield of cowpea by 28.3 % and 25.6 %, respectively, compared with normal conditions. Little research has specifically looked at the impacts of dust on soybeans yield as a result of dust being present on leaves (Gnoinsky et al., 2019). Our results revealed that SPL and HSM had the most significant positive direct effects on seed yield. As a result, these traits were the main responsible for the majority of variation in SY. Therefore, the decrease in SY could be related to the significant reduction in these two traits. The correlation coefficients

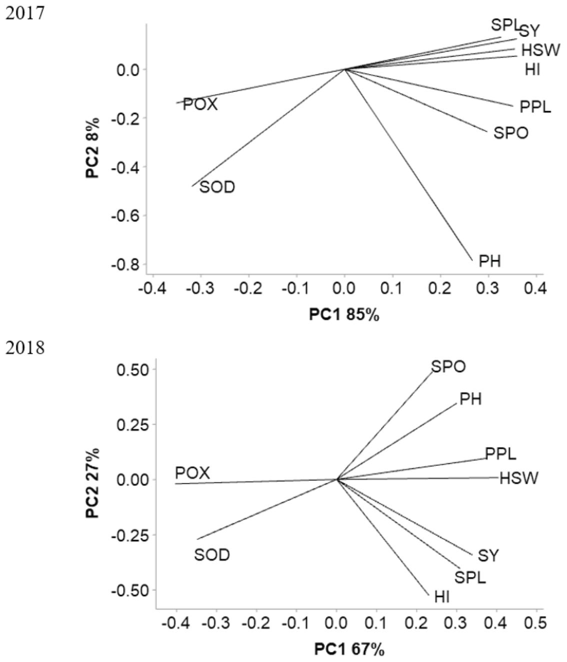


Figure 7: Principal components analysis of the soybean traits under the influence of dust accumulation studied in 2017 (the upper figures) and 2018 (bottom figures)

between SY and yield-related traits confirmed this inference. It has been suggested that under the impact of dust, the acidic secretion of stigma turned into alkaline, a condition that is unfavorable for pollen germination, which leads to poor fertilization and decrease in SPL as well as SPO (Borka, 1981; Sett, 2017). It seems that under the influence of dust, the drop observed in PPL, and HSM was mainly caused by a defect in the plant's photosynthetic system due to clogging of the stomata and, as a result, a decrease in gas exchange. Interference in the gas exchanges between the leaf and the air is one of the most critical consequences of dust accumulation on the leaves. At the same time, the photosynthesis process requires gas exchange through the stomata of the leaves. Thus any disturbance in the stomata pathways can cause problems with photosynthesis (FeleKari et al., 2017). Besides, it was evident that dusty leaves receive less light because of the shading effects due to the deposition of suspended particulate on the leaf surface. Therefore, dust-covered leaves will face a decrease in photosynthesis.

The results of this study indicated that dusted plants had significantly smaller SC compared with control. The reason seems to be the clogging of the stomata above the leaf surface. Dust particles interfere with the mechanism and operation of the stomata, resulting in a significant reduction in gas exchange. Similar to this result, Zia-Khan et al. (2015) reported a substantial decrease in SC of the dusted plants compared to the control. In one study, dusted leaves of *Triticum aestivum* L. and *Pisum sativum* L. had a more number of blocked stomata significantly as compared with control leading to decreased SC (Rahman, 2015).

Results showed that the dusty environment induced the activity of the antioxidant enzymes. In plants, the activity of antioxidant enzymes generally increases under stress conditions. Also, the increase in antioxidant enzyme activity is significantly correlated with the severity of stress (Pilon et al., 2006; Zhang et al., 2014). Antioxidant enzymes work together to eliminate excess reactive oxygen species (ROS), thus protecting the structures and functions of cellular components. SOD is an essential constituent of the antioxidant defense system in plants which. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (Tyagi et al., 2019). Consistent with our results, several studies reported that SOD activity increased in response to dust deposition in plants (Erdal & Demirtas, 2010; Siqueira-Silva et al., 2017; Siqueira-Silva et al., 2016). POD, another antioxidant enzyme, is broadly distributed among plant tissues and plays a significant role in various growth, development, and senescence processes. POD cooperates with SOD to eliminate superoxide and hydrogen peroxide to protect proteins and lipids against ROS (Zhang et al., 2014). The results of this study showed that POD activity increased significantly with the increase in SOD. The induction of the POD activity under dust pollution has also been reported earlier in several tree species (Keller, 1974), some wild dicotyledonous plants including *Chrozophora plicata* A. Juss., and *Croton bonplandianum* R. Br., *Clerodendron inerme* Gaertn; *Solanum torvum* Swartz.; *Calotropis procera* R. Br. (Sarkar et al., 1986), and grapevine (*Vitis vinifera* L.) (Karami et al., 2017).

5 CONCLUSIONS

We found that applying the dust from the vegetative growth stage caused the highest decrease in soybean yield and yield components, and at the same time, led to the highest increase in the antioxidant enzyme activities. Compared to different growth stages, the occurrence of dust at the V3 stage causes plants to be exposed to dust for more extended periods. On the other hand, most crops become increasingly tolerant during later stages of growth (Pirasteh et al., 2014). Therefore, the occurrence of dust at the vegetative stage led to more significant adverse effects on soybean. In conclusion, it was found that the dust accumulation on soybean leaf surfaces reduced stomatal conductance, yield, and yield components. Loss in grains plant^{-1} and one hundred seed mass was the main reason for the reduction in grain yield. More importantly, the results of this study show that yield and yield components were adversely affected by dust deposits during the vegetative period.

6 REFERENCES

Abdullaev, S. F., & Sokolik, I. N. (2020). Assessment of the influences of dust storms on cotton production in Tajikistan. In G. Gutman, J. Chen, G. M. Henebry, & M. Kappas (Eds.), *Landscape Dynamics of Drylands across Greater Central Asia: People, Societies and Ecosystems* (pp. 87-105). Springer International Publishing. https://doi.org/10.1007/978-3-030-30742-4_6

Babu, P. H., Rao, K. N., Jayalalitha, K., & Ali, M. A. (2018). Assessment of different dust pollutants effect on total chlorophyll content, transpiration rate and yield of blackgram (*Phaseolus mungo* L.). *International Journal of Current Microbiology and Applied Sciences, 7*(4), 2890-2896. https://doi.org/10.20546/ijcmas.2018.704.329

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry, 44*(1), 276-287. https://doi.org/10.1016/0003-2697(71)90370-8

Borka, G. (1981). Effect of cement kiln dust on maize plant. *Acta Agronomica Hungarica, 30*, 289-295.

Chaurasia, S., Karwariya, A., & Gupta, A. D. (2014). Impact of cement industry pollution on morphological attributes of wheat (*Triticum* Species) Kodinar Gujarat, India. *Journal of Environmental Science, Toxicology and Food Technology, 8*(7), 84-89. https://doi.org/10.9790/2402-08728489

Doabi, S. A., Afyuni, M., & Karami, M. (2017). Multivariate statistical analysis of heavy metals contamination in atmospheric dust of Kermanshah province, western Iran, during the spring and summer 2013. *Journal of Geochemical Exploration, 180*, 61-70. https://doi.org/10.1016/j.gexplo.2017.06.007

Drack, J. M. E., & Vázquez, D. P. (2018). Morphological response of a cactus to cement dust pollution. *Ecotoxicology and Environmental Safety, 148*, 571-577. https://doi.org/10.1016/j.ecoenv.2017.10.046

Erdal, S., & Demirtas, A. (2010). Effects of cement flue dust from a cement factory on stress parameters and diversity of aquatic plants. *Toxicology and Industrial Health, 26*(6), 339-343. https://doi.org/10.1177/0748233710369235

FeleKari, H., Ghobadi, M. E., Ghobadi, M., Jalali-Honarmand, S., & Saeidi, M. (2017). Effect of dust deposition on yield and yield components of chickpea (*Cicer arietinum* L.) under rain fed and supplemental irrigation conditions in Kermanshah. *Journal of Agroecology, 9*(2), 535-544. https://doi.org/10.22067/jag.v9i2.54549

Glotter, M., & Elliott, J. (2016). Simulating US agriculture in a modern dust bowl drought. *Nature Plants, 3*(1), 16193. https://doi.org/10.1038/nplants.2016.193

Gnoinsky, A., Hargiss, C. L. M., Prischmann-Voldseth, D., & DeSutter, T. (2019). Road dust fails to impact soybean physiology and production. *Agronomy Journal, 111*(4), 1760-1769. https://doi.org/10.2134/agronj2018.10.0640

Hatami, Z., Rezvani Moghaddam, P., Rashki, A., Mahallati, M. N., & Habibi Khaniani, B. (2018). Effects of desert dust on yield and yield components of cowpea (*Vigna unguiculata* L.). *Archives of Agronomy and Soil Science, 64*(10), 1446-1458. https://doi.org/10.1080/03650340.2018.1440081

Hirano, T., Kiyota, M., & Aiga, I. (1995). Physical effects of dust on leaf physiology of cucumber and kidney bean plants. *Environmental Pollution, 89*(3), 255-261. https://doi.org/10.1016/0269-7491(94)00075-O

Hojati, S., Khademi, H., Faz Cano, A., & Landi, A. (2012). Characteristics of dust deposited along a transect between central Iran and the Zagros Mountains. *CATENA, 88*(1), 27-36. https://doi.org/10.1016/j.catena.2011.09.002

Karami, L., Ghaderi, N., & Javadi, T. (2017). Morphological and physiological responses of grapevine (*Vitis vinifera* L.) to drought stress and dust pollution. *Folia Horticulturae, 29*(2), 231–240. https://doi.org/10.1515/fhort-2017-0021

Keller, T. (1974). The use of peroxidase activity for monitoring and mapping air pollution areas. *European Journal of Forest Pathology, 4*(1), 11-19. https://doi.org/10.1111/j.1439-0329.1974.tb00407.x

Lokuruka, M. (2011). Effects of processing on soybean nutrients and potential impact on consumer health: An overview. *African Journal of Food, Agriculture, Nutrition and Development, 11*(4), 5000-5017. https://doi.org/10.4314/ajfand.v11i4.69170

MacAdam, J. W., Sharp, R. E., & Nelson, C. J. (1992). Peroxidase Activity in the Leaf Elongation Zone of Tall Fescue. *II. Spatial Distribution of Apoplastic Peroxidase Activity in*

Genotypes Differing in Length of the Elongation Zone, 99(3), 879-885. https://doi.org/10.1104/pp.99.3.879

Pilon, M., Abdel-Ghany, S. E., Cohu, C. M., Gogolin, K. A., & Ye, H. (2006). Copper cofactor delivery in plant cells. *Current Opinion in Plant Biology, 9*(3), 256-263. https://doi.org/10.1016/j.pbi.2006.03.007

Pirsaheb, M., Zinatizadeh, A., Khosravi, T., Atafar, Z., & Dezfulinezhad, S. (2014). Natural airborne dust and heavy metals: a case study for kermanshah, Western Iran (2005-2011). *Iranian Journal of Public Health, 43*(4), 460-470.

Rahman, J. K. (2015). Response of two crop plants to dust deposition. *ZANCO Journal of Pure and Applied Sciences, 27*(2), 1-6. https://doi.org/10.21271/zjpas.v27i2.144

Sarkar, R. K., Banerjee, A., & Mukherji, S. (1986). Acceleration of peroxidase and catalase activities in leaves of wild dicotyledonous plants, as an indication of automobile exhaust pollution. *Environmental Pollution Series A, Ecological and Biological, 42*(4), 289-295. https://doi.org/10.1016/0143-1471(86)90013-9

Sett, R. (2017). Responses in plants exposed to dust pollution. *Horticulture International Journal, 1*(1). https://doi.org/10.15406/hij.2017.01.00010

Shahsavani, A., Yarahmadi, M., Jafarzade Haghighifard, N., Naimabadie, A., Mahmoudian, M. H., Saki, … Naddafi, K. (2011). Dust storms: Environmental and health impacts [Review Article]. *Journal of North Khorasan University of Medical Sciences, 2*(4), 45-56. https://doi.org/10.29252/jnkums.2.4.45

Sharma, S. B., & Kumar, B. (2015). Effects of stone crusher dust pollution on growth performance and yield status of gram (*Cicer arietinum* L.). *International Journal of Current Microbiology and Applied Sciences, 4*(3), 971-979.

Sharma, S. B., & Kumar, B. (2016). Effects of stone crusher dust pollution on growth performance and yield status of rice (*Oryza sativa*. L). *International Journal of Current Microbiology and Applied Sciences, 5*(5), 796-806. https://doi.org/10.20546/ijcmas.2016.505.080

Singh, N., Shrivastava, R., & Mishra, A. (2018). Influence of leaf dust deposition on chlorophyll content of *Bougainvillea spectabilis* and *Lanatana camara* growing in vicinity of jaypee cement plant, rewa (mp). *International Journal of Information Research and Review, 5*(9), 5685-5688.

Siqueira-Silva, A. I., Pereira, E. G., Lemos-Filho, J. P. d., Modolo, L. V., & Paiva, E. A. S. (2017). Physiological traits and antioxidant metabolism of leaves of tropical woody species challenged with cement dust. *Ecotoxicology and Environmental Safety, 144*, 307-314. https://doi.org/10.1016/j.ecoenv.2017.06.041

Siqueira-Silva, A. I., Pereira, E. G., Modolo, L. V., Lemos-Filho, J. P., & Paiva, E. A. S. (2016). Impact of cement dust pollution on *Cedrela fissilis* Vell. (Meliaceae): A potential bioindicator species. *Chemosphere, 158*, 56-65. https://doi.org/10.1016/j.chemosphere.2016.05.047

Tomar, D., Khan, A. A., & Ahmad, G. (2018). Response of potato plants to foliar application of cement dust. *Tropical Plant Research, 5*(1), 41-45. https://doi.org/10.22271/tpr.2018.v5.i1.007

Tyagi, S., Shumayla, Singh, S. P., & Upadhyay, S. K. (2019). Role of superoxide dismutases (SODs) in stress tolerance in plants. In: S. P. Singh, S. K. Upadhyay, A. Pandey, & S. Kumar (Eds.), *Molecular Approaches in Plant Biology and Environmental Challenges* (pp. 51-77). Springer Singapore. https://doi.org/10.1007/978-981-15-0690-1_3

Walia, K., Aggarwal, R., & Bhardwaj, S. (2019). Leaf dust accumulation and its relationship with biochemical parameters of different plant species growing along national highway-22, India. *International Journal of Chemical Studies, 7*(1), 1386-1390.

Zhang, X., Yin, H., Chen, S., He, J., & Guo, S. (2014). Changes in antioxidant enzyme activity and transcript levels of related genes in *Limonium sinense* Kuntze seedlings under NaCl stress. *Journal of Chemistry, 2014*, 749047. https://doi.org/10.1155/2014/749047

Zia-Khan, S., Spreer, W., Pengnian, Y., Zhao, X., Othmanli, H., He, X., & Müller, J. (2015). Effect of Dust deposition on stomatal conductance and leaf temperature of cotton in Northwest China. *Water, 7*(1), 116-131. https://doi.org/10.3390/w7010116

doi:10.14720/aas.2020.116.1.1775

Review article / pregledni znanstveni članek

Zasnove kmetijsko-okoljskih ukrepov in njihove značilnosti

Tanja ŠUMRADA[1, 2] in Emil ERJAVEC[1]

Received July 10, 2020; accepted September 6, 2020.
Delo je prispelo 10. julij 2020, sprejeto 06. september 2020.

Zasnove kmetijsko-okoljskih ukrepov in njihove značilnosti

Izvleček: Kmetijsko-okoljska plačila (KOP) so instrument kmetijske politike za spodbujanje kmetijskih praks in aktivnosti, ki presegajo z zakonodajo določene osnovne okoljske standarde. Načrtovanje KOP je kompleksen vsebinski, organizacijski in participativni proces. Odločevalci so soočeni z zahtevno nalogo izbora ustreznih zasnov ukrepov, pri čemer razpoložljive možnosti in njihove prednosti in slabosti večinoma niso zbrane in predstavljene na sistematičen način. Namen prispevka je opredeliti tipologijo ukrepov z vidika podlage za plačilo, koordinacije ukrepanja in načina izbora upravičencev. Nadalje prispevek podaja pregled uporabnosti in izvajanja posameznih zasnov ukrepov v praksi, ki temelji na pregledu literature in analizi primerov ukrepov v državah članicah EU in EFTA. V Evropski uniji sodijo KOP med najpomembnejše instrumente na področju varstva okolja in narave, vendar raziskave kažejo, da imajo kljub velikemu vložku proračunskih sredstev razmeroma majhne okoljske učinke. V zadnjih dveh desetletjih zato v evropskih in drugih državah preizkušajo različne nove zasnove KOP, s katerimi skušajo izboljšati njihovo uspešnost pri doseganju okoljskih ciljev in stroškovno učinkovitost. Ukrepi, ki so doslej večinoma temeljili na izpolnjevanju predpisanih praks, dopolnjujejo in v nekaterih državah že nadomeščajo rezultatsko naravnani in podrobneje prostorsko opredeljeni ukrepi. Nekatere evropske države preizkušajo tudi načine, kako kmetijske prakse, ki omogočajo doseganje ciljev s področja varovanja okolja in narave, uveljaviti s skupnim delovanjem kmetovalcev in drugih deležnikov.

Ključne besede: kmetijsko-okoljski ukrepi; rezultatske sheme; skupno delovanje; prostorsko ciljanje; okoljske dražbe; biodiverziteta v kmetijskih ekosistemih; vplivi kmetijstva na okolje; Skupna kmetijska politika

Designs and characteristics of agri-environmental measures

Abstract: Agri-environmental measures (AEM) are an agricultural policy instrument for enhancing of agricultural practices and activities that go beyond the basic environmental standards. Formulation of agri-environmental measures is a complex substantive, organisational and participatory process. Decision-makers are faced with the difficult task of selecting appropriate design of measures, while the available options and their (dis)advantages are in most part not readily available. The purpose of this paper is to outline a typology of possible AEM in terms of the basis for payments, coordination of actions and selection of beneficiaries. Furthermore, the paper provides an overview of the applicability and implementation of particular measures in practice, which is based on a literature review and an analysis of measures in the EU and EFTA Member States. In the European Union, AEM are among the essential instruments in the field of environmental protection and nature conservation. However, research shows that despite a substantial budgetary allocation, AEM have relatively small environmental impacts. Over the last two decades, various new designs of AEM have been tested in an attempt to improve their environmental effectiveness and efficiency. Predominantly management-based measures are thus becoming more result-oriented and more spatially targeted. Some European countries have also piloted the implementation of collective action by farmers and other stakeholders to achieve environmental and nature conservation objectives.

Key words: Agri-environmental measures; result-based payments; collective action; spatial targeting; environmental auctions; farmland biodiversity; environmental impacts of agriculture; Common agricultural policy

---

[1] Univerza v Ljubljani, Biotehniška fakulteta, Ljubljana, Slovenija
[2] Korespondenčni avtor, e-naslov: tanja.sumrada@bf.uni-lj.si

1 UVOD

Eno od temeljnih vprašanj okoljske politike je, kako ekosistemske storitve na podeželskih območjih, na katere vpliva kmetijska raba, učinkovito vpeljati v instrumente javnih, posebej kmetijskih politik (Gerowitt in sod., 2003). Kmetijsko-okoljska plačila (KOP) so instrument Skupne kmetijske politike (SKP) Evropske unije (EU), s katerim si države članice prizadevajo za zmanjšanje negativnih vplivov kmetijstva na okolje in naravo in za ohranjanje ekosistemskih storitev in drugih pozitivnih družbenih koristi kmetovanja. Upoštevajoč nacionalno sofinanciranje je izvedbi KOP v programskem obdobju 2014–2020 v EU namenjenih 26 milijard EUR oziroma 17 % sredstev v okviru politike razvoja podeželja (PRP), kar je primerljivo sredstvom PRP za investicije v kmetijstvo (okrog 23 %) in za podporo območjem z omejenimi dejavniki (okrog 16 %) (OECD, 2017). V številnih državah je letna poraba sredstev za KOP primerljiva ali celo večja od skupnega proračuna, ki je namenjen izvajanju ostalih okoljskih in naravovarstvenih ukrepov, kot je upravljanje zavarovanih območij in omrežja Natura (Batáry in sod., 2015).

Sistemsko financiranje kmetijsko-okoljske politike v obliki KOP v EU poteka od leta 1987, z MacSharryjevo reformo SKP leta 1992 pa je izvajanje KOP postalo obvezno za vse države članice (Buller in sod., 2000). KOP so namenjena izvajanju nadstandardnih okoljskih praks in so utemeljena z načelom »dobavitelj prejme«, po katerem so kmetje kot ponudniki oziroma dobavitelji javnih dobrin upravičeni do finančne kompenzacije za višje stroške oziroma nižje prihodke, povezane z izvajanjem nadstandardnih okoljskih praks (Uthes in Matzdorf, 2013). Kmetijska gospodarstva se zato v KOP vključujejo prostovoljno in s financerjem sklenejo večletno pogodbo, na podlagi katere nato prejemajo letna plačila. Upravljanje KOP, ki od leta 2000 poteka v okviru sedemletnih Programov razvoja podeželja (PRP), si delijo države članice in Evropska komisija (ECA, 2011).

Kljub velikemu vložku proračunskih sredstev v izvajanje KOP rezultati vrednotenj kažejo, da imajo kmetijsko-okoljski ukrepi v EU razmeroma majhne okoljske učinke (npr. ECA, 2011, 2020; Alliance Environment, 2019). Z raziskavami so pokazali, da KOP na lokalni ravni sicer pogosto povečujejo pestrost in pogostost izbranih vrst, vendar na ravni EU doslej niso bili uspešni pri zaustavitvi izginjanja biotske pestrosti v kmetijskih ekosistemih (Batáry in sod., 2011; Gamero in sod., 2017; Kleijn in Sutherland, 2003). Na področju varstva okolja so evalvacije uspešnosti KOP, ki jo razumemo kot uresničevanje zastavljenih ciljev, redkejše, vendar prav tako pogosto niso zaznale pozitivnih učinkov ali pa so bili ti omejeni (Uthes in Matzdorf, 2013). Podobno velja tudi za Slovenijo (Kaligarič in sod., 2019; Slabe-Erker in sod., 2017; Šumrada in sod., 2020a).

Premajhna uspešnost KOP je lahko povezana z neustreznim načrtovanjem, ki se kaže predvsem pri opredelitvi ciljev, zasnovi ukrepov in izračunu višine plačil (ECA, 2011; Poláková in sod., 2011; Šumrada in sod., 2020b). Posledično je lahko pomemben problem tudi nepripravljenost upravičencev za vpis v KOP, saj so lastnosti ukrepov pomemben dejavnik, ki ga kmetje upoštevajo pri odločanju (Lastra-Bravo in sod., 2015). Načrtovanje KOP je zato zahteven proces, saj morajo KOP omogočiti uresničitev različnih, včasih nasprotujočih si okoljskih ciljev in zagotoviti lokalnim razmeram dobro prilagojene ukrepe (Meyer in sod., 2015). Najbolj uspešni kmetijsko-okoljski ukrepi so običajno ciljne sheme *(higer-level, zonal* ali *dark green schemes),* ki so namenjene doseganju ozko opredeljenih okoljskih ciljev in se zato izvajajo na natančno določenih območjih (npr. Newton, 2017). Po drugi strani imajo sheme s splošnim vpisom *(entry-level, horizontal* ali *light green* schemes), ki zasledujejo široko opredeljene cilje in spodbujajo manj zahtevne okoljske prakse na širših območjih, praviloma majhne učinke (ECA, 2020).

Zasnova *(design)* kmetijsko-okoljskega ukrepa je celovit sistem organizacije, ki vključuje njegovo vsebinsko in prostorsko opredelitev, pogoje in način sklepanja pogodbenih razmerij med financerjem in upravičenci ter razdelitev nalog pri upravljanju med institucijami, kot so nadzor, usposabljanje in koordinacija ukrepanja (Saba, 2017). Večina evropskih držav, vključno s Slovenijo, ukrepe trenutno izvaja v obliki individualnih pogodb s kmeti, ki izvedejo predpisane kmetijske prakse (ENRD, 2019). V številnih državah pa so v zadnjih dveh desetletjih preizkušali tudi drugačne zasnove ukrepov, s katerimi so skušali izboljšati uspešnost KOP in njihovo stroškovno učinkovitost. Zahtevnejše zasnove ukrepov gredo tako predvsem v smeri bolj natančne opredelitve upravičenih območij (prostorska ciljnost) (Reed in sod., 2014), spodbujanja koordinacije in sodelovanja med kmeti (Westerink in sod., 2017) in sistema plačil, ki temelji na rezultatih izvajanja ukrepov (Herzon in sod., 2018). Poteka pa tudi preizkušanje novih teoretičnih konceptov, kot sta skupinski bonus (Parkhurst in Shogren, 2007) in okoljska dražba (Schilizzi, 2017).

Raziskave in izkušnje kažejo, da je izvajanje zahtevnejših zasnov ukrepov v praksi povezano s številnimi izzivi (Uthes in Matzdorf, 2013). Sistem plačil, ki temelji na doseganju rezultatov, na primer omogoča večjo ciljnost in stroškovno učinkovitost v primerjavi z ukrepi s predpisanimi praksami, prav tako je lahko bolje sprejet med upravičenci. Po drugi strani pa se je izkazalo, da je uporaba takšnih ukrepov večinoma omejena na okoljske probleme, ki so dobro prostorsko definirani in raziskani in kjer je mogoče opredeliti enostavno merljive kazalnike stanja (Herzon in sod., 2018). Prav tako je treba presoditi, ali morebitni višji transakcijski stroški, ki so povezani z izvajanjem posamezne zasnove, odtehtajo njeno večjo uspešnost (OECD, 2010).

Odločevalci kmetijske politike so tako soočeni z zahtevno izbiro med različnimi zasnovami kmetijsko-okoljskih ukrepov, pri čemer so razpoložljive možnosti pogojene z omejenimi podatkovnimi, finančnimi in kadrovskimi sredstvi, znanje o njihovih prednostih in slabostih pa je pogosto šibko in ni predstavljeno na sistematičen način (OECD, 2010). Cilji tega prispevka so zato:

- opredeliti tipologijo kmetijsko-okoljskih ukrepov,
- izvesti medsebojno primerjavo njihovih značilnosti in
- pregledati izvajanje zahtevnejših zasnov ukrepov v evropskih državah.

Na ta način želimo zapolniti vrzel na tem področju v domačem slovstvu in prispevati k učinkovitejšem snovanju in izvajanju kmetijsko-okoljske politike. Prispevek doprinaša tudi k razvoju slovenske kmetijsko-okoljske terminologije.

2 VIRI IN METODE

Prispevek temelji na pregledu znanstvenih in strokovnih objav (poročila študij, programski dokumenti in zakonodaja), pri čemer smo se osredotočili predvsem na objave iz evropskih držav. Pregled znanstvene literature smo izvedli s sistematično analizo objav v spletni bazi ISI Web of Science, kjer so iskalni kriteriji vključevali ključne besede »agri-environment(al) contract«, »agri-environment(al) measure«, »agri-environment(al) scheme« ali »agri-environment(al) policy« v obdobju od 1970 do 2019 v angleškem jeziku. Strokovna gradiva smo iskali s pomočjo spletnih brskalnikov in na spletnih straneh ključnih raziskovalnih, evropskih in mednarodnih institucij. Na ta način smo pridobili 2.576 objav (na dan 10. 4. 2020), ki smo jih glede na vsebino razvrstili v več podskupin. V raziskavi smo nato obravnavali skupaj 355 objav, ki se nanašajo na načrtovanje, upravljanje in tipe pogodbenih razmerij v okviru kmetijsko-okoljske politike.

Zaradi primerljivosti družbenega in institucionalnega konteksta smo se pri iskanju primerov kmetijsko-okoljskih ukrepov osredotočili na države članice EU in Evropskega združenja za prosto trgovino (EFTA), ki vključuje Islandijo, Lihtenštajn, Norveško in Švico. Drug kriterij je bil, da smo v pregled vključili samo aktualne ali že zaključene primere ukrepov z zahtevnejšimi zasnovami. Slednje pomeni, da smo izločili ukrepe, ki so zasnovani kot preproste sheme s predpisanimi praksami, individualnim načinom sklepanja pogodb in brez posebnega sistema izbora upravičencev. Prav tako v pregled nismo vključili pilotnih ukrepov, ki so bili razviti zgolj za raziskovalne namene. Končni seznam je obsegal 43 primerov ukrepov (priloga).

3 REZULTATI IN RAZPRAVA

V literaturi so objavljene različne opredelitve tipov kmetijsko-okoljskih ukrepov, ki jih avtorji razvrščajo glede na značilnosti, kot so prostorska ciljnost, dolžina pogodb, porazdelitev tveganja in način izbora upravičencev (OECD, 2013, 2015; Vojtech, 2010). Izmed obstoječih tipologij smo izbrali najpogostejše in jih združili v tri ključne značilnosti ukrepov: podlaga za plačilo, koordinacija ukrepanja in izbor upravičencev.

3.1 PODLAGA ZA PLAČILO IN PORAZDELITEV TVEGANJA ZA DOSEGANJE REZULTATOV

3.1.1 Opredelitev in tipologija zasnov

Prvo področje zasnove ukrepov, ki je v zadnjih dveh desetletjih v Evropi najbolj raziskano, temelji na opredelitvi, kako so v kmetijsko-okoljski pogodbi opredeljeni pogoji za dodelitev plačila. Kmet lahko prejme plačilo pod pogojem, da izvede dogovorjene aktivnosti oziroma kmetijske prakse, ali pa plačilo prejme neposredno za rezultate ukrepanja (Herzon in sod., 2018). Na ta način se med financerjem in upravičencem uredi vprašanje porazdelitve tveganja za dosego rezultatov ukrepa. V prvem primeru tveganje nosi predvsem financer, saj prevzame odgovornost za opredelitev takšnih aktivnosti, ki bodo vodile v uresničitev zastavljenih ciljev. Rezultatske sheme po drugi strani upravičencem omogočajo več fleksibilnosti in možnosti za izvirne rešitve pri izvajanju kmetijskih praks, vendar s tem kmetje prevzamejo tudi večjo odgovornost za končno uspešnost ukrepa (Uthes in Matzdorf, 2013).

Raziskovalci so na podlagi celovitega pregleda dosedanjih ukrepov v Evropi opredelili tri glavne tipe zasnov (preglednica 1) (Herzon in sod., 2018):

- V shemi s predpisanimi praksami *(action-based* ali *management-based scheme)* upravičenec prejme plačilo za izvedbo ali opustitev določenih kmetijskih opravil ali drugih aktivnosti, kot so prepoved ali omejitev uporabe gnojil, košnja po določenem datumu ali obnovitvena rez v visokodebelnem sadovnjaku.
- Druga skrajnost je popolnoma rezultatska she-

ma *(pure result-based* ali *outcome-based scheme),* v kateri kmet nima predpisanih nobenih praks, temveč plačilo prejme v primeru, da doseže določen okoljski ali naravovarstveni učinek. Ta se dokaže z dogovorjenim kazalnikom, kot je število rastlinskih vrst na travniku, gnezdeči par ogrožene živalske vrste ali koncentracija nitratov v vodi.

- Tretja možna zasnova je mešana shema *(hybrid scheme),* ki na različne načine združuje elemente obeh zgoraj opisanih zasnov.

3.1.2 Pregled izvajanja v Evropi

Prve raziskave in poskusno izvajanje mešanih in rezultatskih shem se je pričelo v 90. letih. Financirane so bile pretežno iz nacionalnih in regionalnih proračunov in iz zasebnih virov, uspešni pilotni projekti pa so bili nato običajno vključeni v ukrepe Skupne kmetijske politike, kjer se izvajajo in nadgrajujejo še danes. Najdaljše izkušnje s tovrstnimi shemami zasledimo v Nemčiji, Švici, na Nizozemskem in v Skandinaviji (Švedska in Finska), najmanj 10 let pa potekajo tudi na Irskem, v Franciji in Španiji (Herzon in sod., 2018). Po drugi strani so v državah vzhodne in južne Evrope tovrstni pilotski poskusi precej redki, kar je verjetno deloma povezano s pozno uveljavitvijo kmetijsko-okoljskih plačil v naboru instrumentov kmetijske politike (neredko šele po pristopu k EU), deloma pa tudi s specifičnim politično-ekonomskim in kulturnim okoljem (Chobotová, 2013; Sutcliffe in sod., 2015).

Po zadnjem dostopnem pregledu iz leta 2017 je v Evropi potekalo pet popolnoma rezultatskih shem in okrog 20 mešanih shem, od teh večina v različnih nemških deželah (priloga) (Herzon in sod., 2018). Glavnina ukrepov je usmerjenih v doseganje naravovarstvenih ciljev, in sicer v ohranjanje varstveno pomembnih habitatnih tipov in ekstenzivne rabe travišč (predvsem v Nemčiji, Franciji, Švici in na Irskem) ter določenih živalskih vrst (ujede in velike zveri v Skandinaviji in travniške vrste ptic in ujede na Nizozemskem, v Nemčiji in na Irskem). Nekateri ukrepi poleg tega spodbujajo tudi ohranjanje krajinskih elementov (npr. program Burren na Irskem) (Burton in Schwarz, 2013).

Primerov okoljevarstvenih rezultatskih ali mešanih shem je po drugi strani malo tako v Evropi kot tudi drugod po svetu, kar je verjetno povezano z zahtevnostjo določitve ustreznih kazalnikov (Burton in Schwarz, 2013). Sheme na tem področju zato pogosto vključujejo skupno delovanje. Tipičen primer so večinoma lokalno financirani ukrepi za varstvo voda v Nemčiji, kjer je na izbranem območju treba vzdrževati koncentracijo nitratov v vodah pod določeno mejo. V Španiji poteka tudi ukrep za preprečevanje zaraščanja s pašo, ki je namenjen preprečevanju požarov (Herzon in sod., 2018). Na drugih okoljskih področjih je preizkušanje rezultatskih ukrepov

Preglednica 1: Tipologija kmetijsko-okoljskih ukrepov glede na način plačila in porazdelitev tveganja za doseganje rezultatov med financerjem in upravičencem (povzeto po Herzon in sod., 2018, str. 348)
Table 1: Typology of agri-environmental measures according to the basis for payments and risk distribution between financier and beneficiaries in terms of achieving the results (based on Herzon et al., 2018, p. 348)

Tip zasnove	kategorija	glavne značilnosti	osnova za plačilo
shema s predpisanimi praksami	shema, ki podpira določen sistem kmetovanja	Upravičenci morajo izvesti (ali opustiti) predpisane aktivnosti.	izvedba določenega sistema kmetovanja, ki ima znane okoljske ali naravovarstvene učinke (npr. ekološko kmetijstvo)
	shema s predpisanimi praksami	Upravičenci morajo izvesti (ali opustiti) predpisane aktivnosti.	izvedba (ali opustitev) predpisanih praks, ki prispevajo k doseganju okoljskih ali varstvenih ciljev
mešana shema	shema s predpisanimi praksami in možnostjo rezultatskega dodatka	Upravičenci morajo izvesti (ali opustiti) predpisane aktivnosti, spremljanje doseženih učinkov je neobvezno.	izvedba (ali opustitev) predpisanih praks z dodatnim plačilom, če se dosežejo posebni učinki ali rezultati
	rezultatska shema s predpisanimi osnovnimi praksami	Rezultatska shema, v kateri je izvedba (ali opustitev) nekaterih aktivnosti pogoj za vpis v shemo.	okoljski ali naravovarstveni učinki, ki se dokazujejo s predhodno določenimi kazalniki
rezultatska shema	popolnoma rezultatska shema	Shema ne predpisuje izvajanja nobene kmetijske prakse.	okoljski ali naravovarstveni učinki, ki se dokazujejo s predhodno določenimi kazalniki

trenutno še na nivoju raziskav (Colombo in Rocamora-Montiel, 2018; Sidemo-Holm in sod., 2018).

V številnih državah članicah EU potekajo tudi ukrepi, ki so namenjeni ohranjanju genskih virov v kmetijstvu, kot so tradicionalne in avtohtone pasme rejnih živali, in ohranjanju visokodebelnih sadovnjakov in drugih ekstenzivnih trajnih nasadov. Ti so lahko v določenih elementih prav tako rezultatsko usmerjeni, vendar jih avtorji praviloma obravnavajo kot ločeno kategorijo (Herzon in sod., 2018).

3.1.3 Prednosti in slabosti različnih tipov KOP

V Evropski uniji in drugod po svetu prevladujejo sheme s predpisanimi praksami, za kar je verjetno več razlogov. Na tem področju obstaja največ izkušenj in običajno jih je relativno enostavno zasnovati, izvajati in nadzorovati, kar lahko pomeni tudi nižje transakcijske stroške. Kmetje jih lahko lažje sprejmejo, saj je raven tveganja zanje v primerjavi z drugimi možnostmi nižja (Burton in Schwarz, 2013; Vainio in sod., 2019). Po drugi strani je z izvajanjem shem s predpisanimi praksami povezanih več izzivov. Takšni ukrepi namreč močno omejijo fleksibilnost kmetijskih gospodarstev pri iskanju novih, morda bolj stroškovno učinkovitih okoljskih rešitev (OECD, 2010). Poleg tega določitev enotne rabe pogosto prepreči možnost za dinamično prilagajanje kmetovanja vremenskim razmeram, spremembam v ekosistemu in lokalnim razlikam med posameznimi zemljišči (Sabatier in sod., 2012; Uthes in Matzdorf, 2013). Ker se rezultate ukrepanja spremlja le posredno, če sploh, obstaja tudi nevarnost, da države preveč pozornosti namenijo osnovnim kazalnikom učinka, kot je število sodelujočih kmetov ali vključenih površin (Herzon in sod., 2018).

Ključne prednosti rezultatske in mešane sheme zato vključujejo jasnejšo povezavo med plačilom in okoljskimi učinki ter uspešnejše reševanje kompleksnih okoljskih problemov, za katere je težko predpisati enotno kmetijsko prakso (O'Rourke in Finn, 2020). Več fleksibilnosti pri kmetovanju omogoča, da kmetje v izvajanje ukrepa v večji meri vključijo svoje znanje, izkušnje in poznavanje lokalnih razmer, doseganje okoljskih ciljev pa postane bolj vključeno v upravljanje kmetijskega gospodarstva. Kmetje rezultatske sheme zato praviloma dobro sprejmejo, saj se na ta način lahko samostojno odločajo o kmetijskih praksah, s katerimi dosegajo zastavljene okoljske oziroma naravovarstvene cilje. Obenem jih spodbudijo, da prevzamejo del odgovornosti za rezultate in povečajo njihovo poznavanje in razumevanje okoljskih ciljev (Birge in Herzon, 2019).

Rezultatske sheme omogočajo bolj neposreden sistem nadzora in izkazovanja rezultatov ukrepanja in praviloma izboljšajo sodelovanje med različnimi deležniki, saj zahtevajo bolj celosten sistem načrtovanja in decentralizirano upravljanje (O'Rourke in Finn, 2020). Te sheme so običajno tudi bolj ciljne, saj imajo vgrajeno logiko, da kmetje vpišejo zgolj tista zemljišča, ki so varstveno ali okoljsko primerna. To je lahko tudi razlog za njihovo večjo stroškovno učinkovitost, saj se s tem zmanjša t.i. »mrtva izguba« sredstev, ki se sicer porabijo za financiranje neprioritetnih površin (Burton in Schwarz, 2013; Kaligarič in sod., 2019).

3.1.4 Zasnova in uporabnost rezultatskih in mešanih shem

Ključni dejavniki, ki vplivajo na uspešnost rezultatskih in mešanih shem, so dobro definirani okoljski cilji, ustrezni kazalniki, na katerih temeljijo plačila, in naklonjeno družbeno in institucionalno okolje (Herzon in sod., 2018). Večina avtorjev se strinja, da so te sheme najbolj primerne za okoljske probleme, pri katerih je treba zagotoviti nadaljnjo ohranjanje območja v dobrem okoljskem ali naravovarstvenem stanju, kmetje pa imajo dovolj znanja in izkušenj z ustreznimi kmetijskimi praksami. Po drugi strani so običajno manj primerne za območja, kjer je potrebna renaturacija (obnovitev) habitatov ali vzpostavitev povsem novih praks (npr. novih okoljskih tehnologij), s katerimi kmetje nimajo veliko lastnih izkušenj in so zato tudi manj pripravljeni prevzeti tveganje. Prav tako lahko obnova habitatov v intenzivno rabljeni kmetijski krajini traja precej dlje kot običajna kmetijsko-okoljska pogodba (Uthes in Matzdorf, 2013).

Uporaba rezultatskih shem je smiselna, če je možno opredeliti kazalnike, ki so jasno povezani z okoljskimi cilji in so tudi enostavno merljivi. Ključno je, da so kazalniki odvisni predvsem od kmetijskih opravil in drugih aktivnosti, nad katerimi imajo kmetje nadzor v času trajanja pogodbe, vpliv drugih dejavnikov, kot so vremenske razmere, pa je minimalen (Burton in Schwarz, 2013).

Kazalniki so lahko enostavni (npr. pri pticah število gnezdečih parov) ali sestavljeni iz ocene stanja, ki temelji na več kriterijih (npr. popašenost, sestava travne ruše in prisotnost golih tal na travinju). Ukrep je lahko zasnovan tako, da ima samo en mejnik in posledično samo eno višino plačila (npr. določeno število indikatorskih vrst rastlin na travniku), stopenjski sistem z več mejniki in višinami plačil (npr. 6 in 8 ciljnih vrst rastlin na travniku) ali kontinuirani sistem, kjer se plačilo povečuje zvezno (npr. plačilo se do določene meje povečuje za vsako dodatno vrsto indikatorske rastline). Mejniki pri tem ne smejo biti zastavljeni prenizko, saj bi se tako lahko spodbudilo slabšanje stanja na območju. V primeru, da vseh kazalnikov v praksi ni mogoče opredeliti ali izmeriti na enostaven način, je smiselno razmisliti o mešani shemi, v kateri so določene prakse predpisane (Herzon in sod., 2018; Kaiser in sod., 2019).

Ustreznost kazalnikov in sistem preverjanja sta običajno med glavnimi bojaznimi kmetov, ki jih lahko odvrnejo od sodelovanja v ukrepu, če niso ustrezno naslovljene. Pomembno je, da je sistem za kmete enostaven in razumljiv in da imajo na voljo kakovostno svetovalno podporo (Birge in sod., 2017; Wezel in sod., 2018). Vzpostaviti je treba tudi mehanizem za razreševanje sporov med kmetom in nadzorno službo v primeru, da se ne strinjajo z rezultati meritve kazalnikov (Herzon in sod., 2018).

Slednjič je za razvoj tovrstnih shem ključno primerno družbeno in institucionalno okolje. Rezultatske sheme so običajno nastale kot odgovor na kompleksne okoljske probleme, pri katerih prevladujoči centralno vodeni sistem ukrepov z vnaprej predpisanimi praksami ni deloval. Posledično je bilo treba izboljšati znanje in podatkovne podlage za ukrepanje in vzpostaviti bolj decentraliziran sistem upravljanja (O'Rourke in Finn, 2020; Wezel in sod., 2016). V upravljanje ukrepa so zato pogosto vključene regionalne vladne ali nevladne organizacije, ki so lahko zaradi dobrega poznavanja lokalnih razmer bolj kompetentne za tovrstne naloge kot institucije na državni ravni. Ključna elementa, ki omogočata delovanje celotnega sistema, sta zaupanje med posameznimi deležniki, še posebej med financerjem in kmetijskim gospodarstvom, in fleksibilnost, ki omogoča eksperimentiranje in nadgradnjo ukrepa na podlagi pridobljenih izkušenj (Herzon in sod., 2018).

3.2 KOORDINACIJA UKREPANJA IN NAČIN SKLEPANJA POGODB

3.2.1 Opredelitev in tipologija zasnov

Za številne okoljske probleme je značilno, da zadevajo obsežna območja, zato je uspešno upravljanje z naravnimi viri odvisno od usklajenega delovanja večjega števila akterjev. Ohranjanje populacij rastlinskih in živalskih vrst, ki so sposobne dolgoročnega preživetja, je odvisno od zadostne površine prostorsko povezanih habitatov, ki omogočajo migracijo osebkov znotraj in med posameznimi populacijami. Koordinirano ukrepanje je potrebno tudi pri številnih okoljevarstvenih izzivih, kot so varstvo voda pred onesnaženjem z nitrati in fitofarmacevtskimi sredstvi, varstvo tal pred erozijo in blaženje podnebnih sprememb. Sodelovanje več akterjev pa je lahko pomembno tudi, ko kmetijstvo neposredno koristi ekosistemske storitve, zlasti če je za njih značilno, da sodijo med javne dobrine *(public goods)*. Takšni primeri so na primer raba vode za namakanje in ohranjanje opraševalcev in naravnih plenilcev za kmetijstvo škodljivih organizmov (Zavalloni in sod., 2019).

Za uresničevanje večine okoljskih ciljev lahko zadostuje že koordinacija ukrepanja *(coordination)*, s katero se usmerja individualno delovanje posameznih kmetijskih gospodarstev. Spodbujanje sodelovanja *(collaboration)*, ki pomeni aktivno srečevanje, skupno delo in morebitne skupne naložbe kmetov, pa je običajno potrebno v primeru kompleksnih okoljskih problemov, ki vključujejo tudi širšo socialno in ekonomsko problematiko območja (Boulton in sod., 2013).

Najbolj preprost sistem za spodbujanje koordiniranega delovanja so komunikacijske aktivnosti (Preglednica 2), s katerimi se kmete ozavešča o okoljskem problemu in spodbuja k izvajanju aktivnosti. Pravno zavezujoč dogovor v obliki kmetijsko-okoljske pogodbe pa lahko financer sklene neposredno s posameznim kmetom ali pa se ti organizirajo na različne načine, ki segajo od neformalnega projektnega sodelovanja do ustanovitve nove pravne osebe, kot je zadruga ali interesno združenje. Slednja lahko v imenu skupine kmetov sklene pogodbo s financerjem in prevzame določene naloge, kot so priprava skupne vloge in načrta upravljanja, administriranje, izvedba plačil in nadzor (Polman in sod., 2010).

Doseganje skupnih ciljev je mogoče spodbuditi tudi z nadgradnjo individualnih pogodb s skupinskim bonusom *(agglomeration bonus)*. V tem primeru kmet prejme dodatno plačilo, če se v ukrep vključijo zemljišča, ki so v lasti sosednjih kmetov (mejašev) ali pa določen obseg zemljišč na širše opredeljeni prostorski enoti (npr. krajevna skupnost ali vodovarstveno območje). Ukrep pa je lahko zasnovan tudi po načelu »vse ali nič«. To pomeni, da se celotno plačilo oziroma izvajanje ukrepa aktivira le pod pogojem, da se doseže opredeljeni obseg zemljišč na območju, kar se v literaturi imenuje skupinsko plačilo *(agglomeration payment)* (Wätzold in Drechsler, 2014).

Z namenom koordinacije ukrepanja se v proces poleg financerja in kmetov lahko vključijo tudi druge organizacije, ki na lokalni ravni prevzamejo določene naloge pri upravljanju ukrepa, kot so nadzor nad izvajanjem, svetovalna podpora ali sklepanje pogodb v imenu financerja (Polman in sod., 2010). Običajno gre za okoljske nevladne organizacije (NVO), lokalne skupnosti in uprave zavarovanih območij, redkeje pa za privatne akterje (npr. podjetja s koncesijo za oskrbo s pitno vodo) z močnim interesom za doseganje okoljskih ciljev in kapacitetami za koordinacijo kmetov na območju. Na ta način financer upravljanje ukrepa prenese na lokalno raven, kjer je doseganje cilja v skupnem interesu večjega števila deležnikov (OECD, 2013; Westerink in sod., 2017).

Zadnji tip zasnove je oddaja zemljišč v upravljanje. V tem primeru financer ali druga pooblaščena organiza-

Preglednica 2: Tipologija kmetijsko-okoljskih ukrepov glede na način sklepanja pogodb in koordinacijo ukrepanja (prirejeno po OECD, 2013; Polman in sod., 2010; Wätzold in Drechsler, 2014)
Table 2: Typology of agri-environmental measures according to the contracting approach and coordination of actions (based on OECD, 2013; Polman et al., 2010; Wätzold in Drechsler, 2014)

Tip zasnove	kategorija	glavne značilnosti in način sklenitve pogodbe
komunikacijske aktivnosti	informiranje in ozaveščanje	Financer z upravičenci ne sklene formalnih dogovorov, temveč z informiranjem spodbuja izvedbo ukrepov ali pospešuje izmenjavo izkušenj med kmeti.
individualne pogodbe	individualne pogodbe	Individualna pogodba med financerjem in upravičencem brez obveznosti na področju koordiniranega ukrepanja.
	individualne pogodbe s skupinskim bonusom	Individualna pogodba z možnostjo dodatnega plačila, če upravičenci dosežejo določen skupen cilj.
skupno delovanje	skupno delovanje s pomočjo posrednika	Zunanja organizacija (NVO, vladna agencija ipd.) prevzame vlogo posrednika na lokalnem nivoju in v imenu financerja sklepa pogodbe z upravičenci in koordinira njihovo delovanje.
	skupno delovanje s pomočjo skupne organizacije kmetov	Kmetje tvorijo pravno osebo (npr. zadrugo), ki v njihovem imenu sklene pogodbo s financerjem in na podlagi dogovorjenih pravil koordinira njihovo delovanje.
formalni upravljalec	oddaja zemljišč v upravljanje	Financer prevzame lastništvo zemljišč na območju in sklepa individualne ali skupinske pogodbe z upravičenci tako, da ti z zemljišči upravljajo pod določenimi pogoji.

cija postane formalni upravljavec s prevzemom lastništva nad zemljišči, ki so ključna za doseganje okoljskega cilja, in nato pod določenimi pogoji prenese upravljanje na zainteresirane kmete. Pogodba lahko predvideva plačilo najemnine s strani kmeta, brezplačno upravljanje ali pa plačilo storitev kmetu s strani lastnika zemljišč (Polman in sod., 2010). Možno je tudi, da formalni upravljavec s kmeti, ki so lastniki zemljišč na ciljnem območju, zamenja zemljišča v svoji lasti, ki se nahajajo izven ciljnega območja (OECD, 2013). Tipični primeri takšnih upravljalcev so javni skladi in uprave zavarovanih območij, ki imajo v upravljanju kmetijska zemljišča v državni lasti, ali zasebne ustanove, druge NVO in podjetja, ki so odkupila ali drugače pridobila lastništvo nad okoljsko ali naravovarstveno pomembnimi območji (Polman in sod., 2010).

3.2.2 Pregled izvajanja v Evropi

Skupna kmetijska politika v programskem obdobju 2014–2020 omogoča zasnovo ukrepov v obliki skupnega delovanja *(collective action)* (Evropska komisija, 2014). Kljub temu se pogodbe v okviru KOP v večini držav članic EU trenutno sklepajo individualno (ENRD, 2019).

Nizozemska je najbolj znana članica EU, ki je pri zasnovi kmetijsko-okoljskih ukrepov uporabila skupno delovanje s pomočjo organizacij kmetov. Sistem, ki ga razvijajo že več kot 20 let, trenutno temelji na okrog 40 registriranih okoljskih zadrugah ali drugih pravnih osebah, ki so jih ustanovili kmetje in so upravičene do sodelovanja v KOP (Ministry of Economic Affairs, 2016). V drugih državah se organizacije kmetov kot upravičenke v KOP pojavljajo zgolj v redkih primerih ukrepov (npr. v Franciji, italijanski deželi Marke in Veliki Britaniji). V Franciji so za večino KOP vzpostavili prilagojeno zasnovo skupnega delovanja preko posrednika, ki je nekoliko podoben sistemu LEADER. Deležniki s pomočjo lokalnega koordinatorja pripravijo in na javnih pozivih oddajo lokalne kmetijsko-okoljske projekte, ki so nato podlaga za izvajanje ukrepov na posameznih območjih. Lokalni koordinator, ki je odgovoren za njihovo pripravo, kasneje sodeluje tudi pri komuniciranju in svetovanju kmetom (ENRD, 2018, 2019).

V Švici je zanimiv primer zasnove individualnih pogodb s skupinskim bonusom, ki je bil vzpostavljen leta 2001 *(Network bonus scheme)*. Kmetje poleg osnovnega plačila, ki ga prejmejo v sistemu okoljskih kompenzacijskih plačil, lahko prejmejo dodatno plačilo, če sodelujejo v lokalnem projektu mreženja, namenjenem izboljšanju povezljivosti med habitati na območju. Verjetno gre za edini primer tega tipa zasnove v Evropi (Krämer in Wätzold, 2018), čeprav gre glede na način upravljanja morda bolj za primer skupnega delovanja.

Drugi primeri zahtevnejših zasnov kmetijsko-okoljskih ukrepov na področju koordinacije ukrepanja se v Evropi zaenkrat financirajo iz državnih ali lokalnih proračunov in v okviru privatnih shem (priloga) (OECD, 2013). Vsebinsko ukrepi naslavljajo tako okoljevarstvene (zlasti varstvo voda) kot tudi naravovarstvene cilje (ENRD, 2018; OECD, 2013; Westerink in sod., 2017).

3.2.3 Primerjava in uporabnost različnih tipov

Čeprav individualne pogodbe praviloma ne določajo posebnih pravil glede sodelovanja z drugimi kmeti, lahko financer tudi s takšno zasnovo do določene mere koordinira ukrepanje, in sicer z določitvijo pogojev, upravičenih območij in načinom izbora med upravičenci. Ker pa večina odločitev glede vpisa zemljišč ostane v rokah posameznih upravičencev, so te možnosti običajno omejene (Polman in sod., 2010). Okoljske probleme, ki zahtevajo koordinirano ukrepanje, je zato z individualnimi pogodbami praviloma mogoče rešiti le, če se v ukrep vključi veliko število kmetijskih gospodarstev, s čimer se na območju doseže kritično mejo površin. Da bi dosegli slednje, morajo biti tovrstni ukrepi za kmete še posebej atraktivni (Franks in Emery, 2013).

Skupno delovanje v primerjavi z individualnimi pogodbami načeloma omogoča uspešnejšo koordinacijo ukrepanja na ravni problemskega območja, zato se lahko s takšnim pristopom doseže tudi kompleksnejše okoljske cilje (Leventon in sod., 2017). Na strani upravičencev in financerja se lahko zmanjša administrativno breme in transakcijski stroški, saj posredniške ali skupne organizacije kmetov združujejo določene naloge, povezane s sodelovanjem in upravljanjem (Prager, 2015). Poleg tega je lahko zaradi ekonomije obsega skupni prehod na okoljsko sprejemljivejše kmetijske prakse stroškovno učinkovitejši kot individualne naložbe (OECD, 2013).

Skupno delovanje praviloma spodbudi izmenjavo znanja in sodelovanje med kmeti, s čimer se poveča socialni kapital skupnosti, pospeši nastajanje inovacij in izboljša pogajalski položaj in sodelovanje kmetov ter drugih deležnikov pri snovanju ukrepov (Westerink in sod., 2017). V tovrstnih sistemih se praviloma vzpostavi intenzivnejši dialog med kmeti in drugimi deležniki, s čimer se poveča možnost za dosego soglasja o skupnih ciljih in oblikovanje lokalnim razmeram bolje prilagojenih ukrepov (ENRD, 2018). Kljub številnim oviram za sodelovanje (Riley in sod., 2018) raziskave kažejo, da so kmetje načeloma pripravljeni na skupno delovanje, če je to zasnovano na preprost način (McKenzie in sod., 2013). Takšna zasnova pa verjetno najbolje deluje pod pogojem, da je formiranje skupnih organizacij kmetov tudi pogoj za sodelovanje v ukrepu (Franks in Emery, 2013).

Vzpostavitev skupnega delovanja v kmetijsko-okoljskih ukrepih je zahteven proces, ki glede na obstoječe izkušnje za uveljavitev potrebuje več let (Mills in sod., 2011; Westerink in sod., 2017). Pred izbiro takšne zasnove je zato treba dobro raziskati okoljski problem in socio-ekonomske značilnosti območja. Skupno delovanje je smiselno spodbujati, ko razsežnosti okoljskega problema presegajo zmožnosti ukrepanja na ravni posameznih kmetijskih gospodarstev in kadar med lokalnimi deležniki obstaja interes za skupno reševanje problema. Uspešnost te zasnove je namreč močno povezana s časom in sredstvi, ki so jih deležniki pripravljeni in zmožni investirati v njegovo vzpostavitev in upravljanje (Prager, 2015). Več avtorjev izpostavlja tudi pomen lokalnega pobudnika, ki sproži in kasneje vodi proces povezovanja, na območju pa deluje kot motivator in stičišče informacij (ENRD, 2018; Rac in sod., 2020).

Za vzpostavitev takšnih shem je potrebno stabilno in podporno politično okolje, saj v primeru nenehnega spreminjanja razvojnih usmeritev in instrumentov kmetijske politike zaupanja med deležniki ni mogoče vzpostaviti. Ker je treba vzpostaviti sodelovanje med akterji in preseči različne ovire, imajo ti ukrepi na začetku običajno višje transakcijske stroške kot v fazi zrelosti, zato so vsaj v začetni fazi vzpostavljanja koristni dodatni podporni ukrepi, ki takšne stroške nadomestijo (OECD, 2013).

3.3 CILJANJE IN IZBOR UPRAVIČENCEV

3.3.1 Opredelitev in tipologija zasnov

Kmetijsko-okoljski ukrepi naj bi bili zasnovani čim bolj stroškovno učinkovito, kar pomeni, da se okoljski cilj skuša doseči s čim manj sredstvi, hkrati pa je razmerje med mejno koristjo in mejnimi stroški njihove izvedbe čim bolj uravnoteženo. Na ravni posameznih kmetijskih gospodarstev to pomeni doseganje okoljskih učinkov z minimalnimi stroški, na ravni območij pa, da so okoljski cilji doseženi z minimalnimi stroški za kmetijska gospodarstva in z optimalno porazdelitvijo okoljskega napora med njimi (OECD, 2010).

Iz slednje zahteve izhaja ključni problem načrtovanja stroškovno učinkovitih KOP, saj se kmetijska gospodarstva med seboj razlikujejo tako po vplivu na okolje kot tudi po stroških, ki jih imajo z izvajanjem okoljskih ukrepov. Nekatera kmetijska gospodarstva imajo na primer svoja zemljišča neposredno ob vodotokih, zato je njihov vpliv na kakovost voda večji kot pri kmetijskih gospodarstvih z bolj oddaljenimi zemljišči. Njihov napor, ki je potreben za doseganje enakega vpliva na okolje, je zato večji, kar je smiselno upoštevati pri razporejanju zahtevnosti obvez v različnih ukrepih. Po drugi strani se kmetijska gospodarstva razlikujejo tudi v svoji proizvodni in ekonomski strukturi, zato imajo z izvedbo istega ukrepa lahko različne stroške. Če torej financer na območju razpiše ukrep z enakimi obveznostmi in plačilom za vsa kmetijska gospodarstva, bo najverjetneje prišlo do nepotrebne obremenitve kmetij z majhnim vplivom na okolje in preplačila tistih, ki imajo nizke stroške z izvajanjem ukrepa. Po drugi strani kmetijska gospodarstva z okoljsko najbolj problematičnimi zemljišči in višjimi stroški v ukrepu najverjetneje sploh ne bodo sodelovala, saj zanje ne bo dovolj privlačen (OECD, 2010).

V procesu snovanja kmetijsko-okoljskih ukrepov je zato treba nasloviti prostorsko in ekonomsko heterogenost kmetijskih gospodarstev, kar je še posebej zahtevno, ker med financerjem in upravičenci obstaja informacijska asimetrija. Financer namreč običajno pri odločanju nima tako podrobnih podatkov o posameznem kmetijskem gospodarstvu kot lastnik, ki je o stanju svoje kmetije in stroških, povezanih z njenim upravljanjem, praviloma bolje informiran. Določene informacije so tako financerju skrite, zato lahko pri izboru upravičencev pride do napačne izbire *(adverse selection),* saj se bodo za sodelovanje odločala predvsem kmetijska gospodarstva, pri katerih se obveznosti v ukrepu ne razlikujejo bistveno od njihovega obstoječega načina pridelave in imajo torej z ukrepom nizke stroške (Latacz-Lohmann in Schilizzi, 2005). To z vidika doseganja okoljskih ciljev ni zaželjeno, saj takšen ukrep nima veliko dodatnosti *(additionality),* tj. primerljive učinke bi lahko dosegli tudi brez ukrepanja (FAO, 2011).

Pri snovanju kmetijsko-okoljskih ukrepov se kot odgovor na problem heterogenosti kmetijskih gospodarstev uporablja različne načine ciljanja *(targeting)* in izbora upravičencev *(enrolment screening).* S tem se opredeli območja, kmetijska gospodarstva ali zemljišča, ki so pomembni za doseganje okoljskega cilja, in nato diferencira zahtevnost obveznosti in višino plačil glede na njihove značilnosti. Financer lahko to naredi z zbiranjem podrobnih podatkov o stanju okolja na območju in ekonomiki posameznih kmetijskih gospodarstev, vendar je to povezano z dodatnimi stroški. Druga možnost je vzpostavitev mehanizmov, s katerimi se kmete spodbudi, da ob vpisu v ukrep tovrstne informacije razkrijejo sami (preglednica 3) (OECD, 2010).

Prostorsko ciljanje *(spatial targeting)* izvajanje ukrepov usmerja na najbolj ranljiva ali pa na najbolj primerna območja in zemljišča, s čimer naj bi se doseglo čim boljše razmerje med okoljskimi učinki in stroški (Uthes in sod., 2010). Ciljanje lahko temelji na opredelitvi posebnih geografskih območij ali naravnih značilnosti (npr. npr. zavarovana območja, visokogorski, visokogorski pašniki ali povodje vodotoka), strukturnih značilnosti kmetijskih gospodarstev ali tipov pridelave *(structural targeting)* (npr. ekološko kmetovanje, prašičjereja ali trajni nasadi na terasiranih pobočjih) ali reševanju drugih specifičnih potreb, ki jih je mogoče prostorsko dobro opredeliti (npr. območje pojavljanja ogrožene vrste) (Evropska komisija, 2014; OECD, 2015). Poleg prostorske omejitve ukrepanja se lahko pri ciljanju upoštevajo tudi pričakovani stroški izpolnjevanja obveznosti. Namesto enotnega plačila na vseh ciljnih območjih se tako lahko uvede heterogena plačila, ki upoštevajo razlike v razmerah na posameznih območjih ali med njimi (Wätzold in Drechsler, 2005).

Financer lahko okoljsko prioritetnost in stroške izvajanja ukrepov na posameznih zemljiščih pridobi tudi

Preglednica 3: Tipologija kmetijsko-okoljskih ukrepov glede na način ciljanja in izbora upravičencev
Table 3: Typology of agri-environmental measures according to the targeting approach and enrolment screening

Tip zasnove	kategorija	glavne značilnosti
shema s splošnim vpisom	splošni vpis upravičencev	Ukrep se izvaja na širokem ali celotnem območju delovanja financerja. Postopek izbora upravičencev ni predviden, lahko pa je posredno vključen z opredelitvijo obveznosti v ukrepu.
prostorsko ciljanje	pričakovani okoljski učinki	Opredelijo se prioritetna območja, na katerih je mogoče sodelovati v ukrepu, ali tipi kmetijskih gospodarstev, za katere se pričakuje, da lahko močno prispevajo k uresničitvi okoljskega cilja. Plačilo je enotno za vse upravičence.
	pričakovani stroški izpolnjevanja obveznosti	Opredelijo se upravičena območja ali tipi kmetijskih gospodarstev. Pri izračunu višine plačil se upošteva različnost razmer na ali med posameznimi območji ali skupinami kmetijskih gospodarstev, zato so plačila heterogena.
sistem izbora med upravičenci	vsebinski izbor	Zainteresirani upravičenci podajo ponudbe (npr. okoljske programe za kmetijsko gospodarstvo ali območje), ki jih financer presodi na podlagi vsebinskih kriterijev. Izbere se ponudbe, ki dosežejo minimalno število točk ali po vrsti do porabe sredstev.
	okoljske dražbe	Zainteresirani upravičenci oddajo ponudbe, ki vključujejo tudi ponudbeno ceno. Financer izbere najbolj ugodne ponudnike glede na razmerje med okoljskim učinkom in ceno.

neposredno od kmetijskih gospodarstev s pomočjo izbora ponudb upravičencev. V tem primeru zainteresirani upravičenci na dogovorjen način oddajo ponudbe, med katerimi nato financer izbira glede na razpoložljiva sredstva. Če izbor temelji zgolj na vsebinskih oziroma okoljskih kriterijih *(performance-based screening),* so za financiranje izbrane ponudbe, ki presežejo določen prag kakovosti (npr. število točk), ali pa se glede na rezultate ocenjevanja oblikuje lista upravičencev, ki so izbrani po vrsti do porabe sredstev (OECD, 2010).

Druga možnost je uvedba okoljske ali naravovarstvene dražbe *(environmental* ali *conservation auction),* na kateri se pri izboru najbolj ugodnih ponudnikov poleg okoljskih učinkov upošteva tudi ponudbeno ceno. Upravičenec mora zato pri oddaji ponudbe tehtati med višino prejetega plačila in verjetnostjo izbora, saj se v primeru previsoke ponudbene cene lahko zgodi, da izpade iz financiranja (Uthes in Matzdorf, 2013). Okoljske dražbe so lahko zasnovane kot razlikovalne zaprte dražbe *(discriminatory sealed-bid auction),* na katerih so izbrani najbolj ugodni ponudniki, ki prejmejo plačilo v skladu s svojo podano ponudbo. Druga možnost so zaprte dražbe z enotno ceno (*uniform-price sealed-bid auction),* ki potekajo tako, da vsi izbrani ponudniki prejmejo enotno plačilo, ki je enako bodisi najnižji izbrani bodisi najvišji zavrnjeni ponudbeni ceni (Latacz-Lohmann in Schilizzi, 2005).

Pri zasnovi okoljskih dražb so pomembne tudi druge značilnosti. Dražba ima lahko vnaprej omejen obseg sredstev, ki so na voljo za ukrep *(budget-constrained auction),* ali pa se ponudnike izbira dokler se ne doseže določen okoljski cilj ali ciljna vrednost *(target-constrained auction).* Financer se mora tudi odločiti, ali bo opredelil »pridržano ceno« *(reserved price),* ki jo razumemo kot najvišjo možno plačilo na enoto okoljskega učinka ali na površino, ki jo je pripravljen plačati, in ali bo to informacijo razkril pred oddajo ponudb (Latacz-Lohmann in Schilizzi, 2005). Vse omenjene značilnosti vplivajo na vedenje ponudnikov, zato je treba pravila dražbe prilagoditi posameznemu okoljskemu problemu in družbenem okolju.

3.3.2 Pregled izvajanja v Evropi

V državah članicah EU so različne oblike ciljanja ukrepov uveljavljen pristop k snovanju kmetijsko-okoljskih ukrepov (Uthes in Matzdorf, 2013). Poglobljenih vrednotenj in raziskav na tem področju ni, je pa Evropska mreža za podeželje (ENRD, 2019) v preliminarnem pregledu izvajanja KOP v programskem obdobju 2014–2020 ocenila, da najbolj ciljane ukrepe izvajajo v petih državah članicah (Estonija, Francija, Irska, Nizozemska in Velika Britanija). Enajst držav članic je uporabilo predvsem različne oblike posrednega ciljanja (npr. s pomočjo opredelitve prioritetnih območij), medtem ko so za deset držav članic, vključno s Slovenijo, ocenili majhno ciljnost ukrepov, saj se ti praviloma izvajajo na območju celotnega ozemlja države. Ciljanje so države običajno izvedle z opredelitvijo ciljnih območij za reševanje specifičnih okoljskih problemov, geografskih območij izvajanja in upravičenih tipov kmetijskih gospodarstev.

Države članice morajo razlike med posameznimi območji in proizvodnimi praksami upoštevati tudi v izračunih višine plačil za ukrepe ali v nasprotnem primeru utemeljiti, zakaj to ni potrebno (Evropska komisija, 2014), vendar pregled, v kolikšni meri in na kakšen način države v izračunih upoštevajo pričakovane stroške izpolnjevanja obveznosti, ni na voljo.

Nekatere države članice EU so vzpostavile določene kriterije za izbor upravičencev, ki so najpogosteje vezani na območja Natura 2000 in območja, ki so v skladu z Okvirno vodno direktivo opredeljena kot občutljiva zaradi onesnaževanja z nitrati (ENRD, 2019). Na Malti imajo tako pri izboru upravičencev prednost tisti kmetje, ki se nahajajo na območjih Natura 2000, ki se prijavijo kot skupina kmetov in ki jih potrdi organizacija, ki je na lokalni ravni odgovorna za doseganje okoljskih ciljev problemskega območja. Podobno poteka tudi v nekaterih italijanskih deželah, na Portugalskem pa imajo prednost kmetje, ki se udeležijo usposabljanj. V nekaterih drugih državah se izbor upravičencev s tovrstnimi kriteriji aktivira le v primeru, da pride do pomanjkanja sredstev (Ciper, Francija, Grčija in nekatere italijanske dežele).

Uporaba okoljskih dražb v kmetijsko-okoljskih ukrepih je v evropskem prostoru razmeroma redka. Sheme, ki vključujejo veliko število površin in upravičencev, se izvajajo samo v ZDA *(Conservation Reserve Program)* in Avstraliji, medtem ko je v Evropi uporaba dražb omejena na manjše programe in pilotne sheme v Nemčiji, Veliki Britaniji in na Finskem (Iho in sod., 2014; Lundberg in sod., 2018; Rolfe in sod., 2017).

3.3.3 Primerjava in uporabnost različnih tipov

Prostorsko ciljanje in izbor upravičencev je v okviru kmetijsko-okoljskih ukrepov smiselno uporabiti, ko obstaja opazna heterogenost med območji ali med upravičenci v smislu njihovega vpliva na okolje in stroškov z izvajanjem ukrepov (FAO, 2011). V takšnih primerih so tovrstne zasnove lahko bolj stroškovno učinkovite, poleg tega imajo ukrepi z enotnimi plačili lahko tudi bistveno manjše okoljske učinke (Armsworth in sod., 2012).

Po drugi strani več avtorjev opozarja na problem transakcijskih stroškov, saj so za izvajanje zahtevnejših zasnov običajno potrebne podrobnejše podatkovne zbirke in s tem povezani dodatni stroški za administracijo ukrepa (Palm-Forster in sod., 2016; Uthes in sod., 2010). Prevelika diferenciacija med kmetijskimi gospodarstvi lahko povzroči tudi negativen odziv upravičencev, saj okoljsko uspešnejša gospodarstva v takšnem sistemu lahko prejemajo nižja plačila (Uthes in Matzdorf, 2013). Vpeljava ciljanja ali sistema izbora običajno do določene mere spremeni porazdelitev sredstev med prejemniki, kar lahko povzroči trenja med deležniki (Uthes in sod., 2010). Problematična pa je lahko tudi preveč toga opredelitev upravičenih območij, še posebej če so v ozadju premalo natančni podatki ali zbirke z veliko napakami (Uthes in Matzdorf, 2013).

Izbor med upravičenci je smiselno uporabiti takrat, ko zanimanje upravičencev za sodelovanje v ukrepu presega razpoložljiva sredstva, kar še posebej velja za okoljske dražbe (OECD, 2010). Prednosti dražbe v primerjavi z ukrepi z enotnim plačilom so, da bolje naslavljajo problem informacijske asimetrije, saj kmetje ob oddaji ponudbe predlagajo tudi višino plačila, za katero ocenjujejo, da je ustrezna za njihovo kmetijo, seveda pod pogojem, da je dražba ustrezno zasnovana. Prav tako so bolj stroškovno učinkovite, ker z omogočanjem konkurence ponudnike spodbujajo, da ponudijo ceno, ki je bližje njihovim oportunitetnim stroškom, zato se zmanjša izguba sredstev. Prednost dražb je lahko tudi v njihovi večji družbeni sprejemljivosti, saj sredstva dobijo najbolj ugodni ponudniki (Latacz-Lohmann in Schilizzi, 2005).

Burton in Schwarz (2013) omenjata, da bi okoljske dražbe v rezultatskih shemah lahko uspešno naslovile problem izračuna plačil. Kljub usmerjenosti rezultatskih shem v nagrajevanje rezultatov namreč ta večinoma še vedno temelji na standardiziranem pristopu ocene višjih stroškov oziroma nižjih prihodkov zaradi izvedbe nadstandardnih kmetijskih praks. KOP so namreč v skladu z leta 1994 sprejetim Sporazumom o kmetijstvu Svetovne trgovinske organizacije (WTO) uvrščena v t.i. zeleno škatlo *(green box)* kmetijskih podpor, za katere velja, da naj bi povzročale minimalno izkrivljanje mednarodnih trgov (Huige idr. 2010). Omenjeni sporazum okoljska plačila opredeljuje kot nadomestilo za dodatne stroške in izpad dohodka, ki nastanejo zaradi vključitve proizvajalcev v državne okoljske ali naravovarstvene programe. To načelo je zato v okviru SKP osnova za izračun KOP, ki po trenutno veljavni zakonodaji lahko vključujejo nadomestilo za dodatne stroške, izpad dohodka in transakcijske stroške, ki so povezani z izvajanjem in sodelovanjem v ukrepu, v določenih primerih pa tudi oportunitetne stroške (Evropska komisija 2014).

Okoljske dražbe načeloma niso primerne za okoljske probleme, ki se pojavljajo na majhnih območjih, saj je ključno, da se zagotovi zadostno število ponudb in posledična konkurenca. Slabost okoljskih dražb je strateško vedenje ponudnikov, saj se kmetijsko-okoljske pogodbe tipično sklepajo za obdobje nekaj let. Dražbe je zato treba ponavljati, s čimer imajo kmetje možnost, da se učijo iz izkušenj s predhodnimi krogi in ustrezno prilagajajo svoje ponudbe. S tem se zmanjšuje stroškovna učinkovitost sheme, zato naj financer ne bi razkrival podatkov o ponujenih zneskih ter številu oddanih in izbranih ponudb, prav tako je smiselno v vsakem krogu nekoliko spremeniti pravila, s čimer se ohranja negotovost pri ponudnikih (OECD, 2010). Pomembne so tudi strukturne razlike, saj so bili v pilotni dražbi na Finskem za sodelovanje bolj zainteresirani profesionalni kmetje iz večjih kmetijskih gospodarstev, manj pa starejši in manj izobraženi kmetje (Grammatikopoulou in sod., 2012).

4 SKLEPI IN PRIPOROČILA ZA PRIHODNJO KMETIJSKO-OKOLJSKO POLITIKO

4.1 SNOVANJE KMETIJSKO-OKOLJSKIH UKREPOV IN DEJAVNIKI USPEHA

Uveljavljanje strateškega načrtovanja Skupne kmetijske politike po letu 2021 v državah članicah EU vključuje tudi pripravo celovitega načrta ukrepanja na področju varstva okolja in narave in zahteva nov razmislek o vsebini in zasnovi kmetijsko-okoljskih ukrepov (Evropska komisija, 2018). Načrtovanje KOP je zahteven proces, v katerem je treba hkrati naslavljati različne okoljske cilje in upoštevati heterogenost naravnih, ekonomskih in socialnih razmer po območjih in kmetijskih gospodarstvih.

Kmetijsko-okoljske ukrepe je smiselno oblikovati, ko doseganje okoljskih ciljev zahteva prilagoditev kmetijskih praks v obsegu, ki presega osnovne okoljske standarde, in ko (višji) stroški, ki so povezani z določenim načinom kmetovanja, niso ustrezno ovrednoteni na trgu. Ključni kriteriji pri izboru ustrezne zasnove ukrepa vključujejo (Herzon in sod., 2018; OECD, 2010):

- okoljsko uspešnost (zmožnost ukrepa, da bo dosegel zastavljene cilje),
- stroškovno učinkovitost (razmerje med koristjo in stroški ukrepa),
- kapacitete institucij, podatkovna podprtost in obseg transakcijskih stroškov, povezanih z upravljanjem ukrepa,
- informiranost in usposobljenost upravičencev,
- posredne koristi in stroške ukrepanja na druge cilje kmetijske in okoljske politike in
- pravičnost v smislu poštene porazdelitve koristi in stroškov med proizvajalci, potrošniki in davkoplačevalci.

V zadnjih dveh desetletjih so države OECD testirale

Preglednica 4: Uporabnost izbranih tipov kmetijsko-okoljskih ukrepov
Table 4: Applicability of the selected types of agri-environmental measures

Rezultatske in mešane sheme	Skupno delovanje	Sistem izbora med upravičenci
• potrebno je nadaljnje ohranjanje ugodnega stanja območja (kmetje imajo izkušnje s primernimi kmetijskimi praksami), • mogoče je natančno opredeliti okoljske cilje in izbrati enostavno merljive kazalnike, • med deležniki je mogoče vzpostaviti dovolj zaupanja, da je odločanje o izvedbi ukrepov lahko deloma prepuščeno kmetom, • kmetje so pripravljeni sprejeti del tveganja za doseganje rezultatov.	• za uspešno naslavljanje okoljskega problema je potrebno koordinirano delovanje večjega števila kmetov, • lokalni deležniki imajo interes in kapacitete za skupno reševanje problema in se strinjajo, da je to najbolj primeren pristop, • obstaja dolgoročno stabilno in podporno politično okolje, ki omogoča vzpostavitev povezav in zaupanja med akterji.	• upravičenci se med seboj opazno razlikujejo po vplivu na okolje in stroških, ki jih imajo z izvajanjem ukrepa, • interes za sodelovanje je večji od razpoložljivih sredstev, • območje in število interesentov je dovolj veliko za nastanek konkurence med ponudniki, • financer težko pridobi podatke o upravičencih, zato je podrobno načrtovanje ukrepov povezano z velikimi stroški, • obstaja politična podpora izbiranju med upravičenci (plačila ne dobijo vsi, temveč samo tisti, ki najbolje izpolnjujejo kriterije).

različne zasnove ukrepov, s katerimi so skušali izboljšati njihovo stroškovno učinkovitost in uspešnost pri doseganju ciljev. V prispevku smo na podlagi pregleda literature in analize izbranih primerov ukrepov, ki se izvajajo v Evropi, razvili tipologijo kmetijsko-okoljskih ukrepov in analizirali njihove značilnosti, ki jih v zgoščeni obliki predstavljamo v Preglednici 4. To omogoča sistematičen pregled možnosti, ki so na voljo, in pripravo priporočil za načrtovanje prihodnje kmetijsko-okoljske politike.

Kmetijsko-okoljski ukrepi, ki se trenutno izvajajo v Evropi, so zelo raznoliki. Na podlagi pregleda literature in analize primerov v tem prispevku ocenjujemo, da je uspešnost ukrepa odvisna predvsem od izbora ustrezne zasnove in prilagojenosti lokalnemu okolju (Plieninger in sod., 2012). Po drugi strani analize kmetijsko-okoljskih ukrepov, še posebej tistih s kompleksnejšimi zasnovami, kažejo, da imajo uspešni ukrepi nekatere skupne značilnosti, ki so se postopoma oblikovale. Te so primerljive ne glede na izbrani tip zasnove, zato jih je mogoče posplošiti v ključne dejavnike uspešnosti kmetijsko-okoljskih ukrepov (Herzon in sod., 2018; Meyer in sod., 2015; O'Rourke in Finn, 2020; Westerink in sod., 2017; Zilans in sod., 2019):

- Dobra raziskanost in poglobljeno razumevanje okoljskega problema: Uspešni ukrepi se običajno razvijejo na območjih z dobro raziskanim ekološkim ali okoljskim ozadjem problema in poglobljenim razumevanjem družbenih značilnosti lokalnega okolja.
- Kmet v središču načrtovanja: Končni upravičenec je v uspešnih ukrepih manj administrativno obremenjen, ima več možnosti odločanja glede načina izvedbe in bolje razume sistem in pomen ukrepanja.
- Zaupanje med deležniki in decentralizacija odločanja: Za vzpostavitev ukrepov je potrebno zaupanje med ključnimi deležniki in njihova vključitev v odločanje. V prvi vrsti gre za zaupanje med financerjem in kmeti, pomembno pa je tudi sodelovanje med kmeti in med različnimi deležniki, ki so vključeni v zasnovo in upravljanje ukrepa.
- Vključitev lokalnih in drugih deležnikov v načrtovanje in upravljanje: Pobudniki inovativnih ukrepov so pogosto zunanji deležniki, ki imajo dolgoročen interes za rešitev problema in kapacitete za sodelovanje pri upravljanju ukrepa.
- Prilagodljivo upravljanje in stabilna podpora politike: Kompleksni in uspešni kmetijsko-okoljski ukrepi nastajajo več desetletij, zato je potrebna dolgoročna podpora politike. Prepoznamo jo v strateških podpornih ukrepih, kot je spodbujanje ciljnih raziskav, pilotnih preizkusov in delovanja lokalnih organizacij, in vzpostavitvi odprtega okolja za preizkušanje novih rešitev in nadgradnjo ukrepov s pridobljenimi izkušnjami.

4.2 PRIPOROČILA ZA PRIHODNJE RAZISKAVE

Večina ukrepov z zahtevnejšimi zasnovami trenutno poteka v gospodarsko razvitejših državah v severnem in zahodnem delu Evrope (ENRD, 2019; Herzon in sod., 2018). Tu se pogosto izvajajo na območjih z razmeroma razvitim kmetijstvom in v politično-ekonomskem okolju, kjer so varstvo okolja in narave pomembne družbene teme, zato obstaja tudi politična volja in nenehno prizadevanje za izboljšanje javnih politik na tem področju. Kljub prednostim novih zasnov KOP zato ostaja odprto vprašanje, ali bodo zasnove, ki temeljijo na rezultatskem pristopu, skupnem delovanju ali zahtevnejših sistemih izbora upravičencev, uspešne tudi v državah vzhodne in južne Evrope, vključno s Slovenijo (Herzon in sod., 2018; Vesterager in sod., 2016). Za številna območja v tej regiji so namreč značilne specifične razmere, ki vključujejo razdrobljeno zemljiško strukturo, majhna, ostarela in polprofesionalna kmetijska gospodarstva, slabo raziskanost okoljske problematike ter neizkušenost in pomanjkanje kapacitet ključnih deležnikov za zahtevnejše oblike kmetijsko-okoljskih ukrepov (Gorton in sod., 2009; Sutcliffe in sod., 2015).

Uspešnost reševanja okoljskih ukrepov v kmetijskih ekosistemih je močno odvisna od usklajenega delovanja različnih deležnikov. Spreminjanje vzorcev sodelovanja med kmetijskimi gospodarstvi in med drugimi akterji na podeželju je kompleksen proces, ki se v Evropi dogaja že več desetletij (Riley in sod., 2018). Kmetijsko-okoljski ukrepi in drugi instrumenti kmetijske politike, ki se praviloma izvajajo v obliki individualnih pogodb, teh trendov do sedaj večinoma niso posebej naslavljali in izolirano delovanje kmetijskih gospodarstev verjetno še dodatno spodbujajo. Prihodnje raziskave in preizkušanje ukrepov bi zato moralo bolje nasloviti ključne ovire za sodelovanje, preizkušanje podpornih instrumentov in spodbujanje lokalnih organizacij, ki bi bile pooblaščene za koordinacijo akterjev (Leventon in sod., 2017).

Ugodno podporno institucionalno okolje, v katerem poteka odločanje in načrtovanje KOP, je ključen predpogoj za razvoj bolj uspešnih ukrepov, vendar je to področje relativno slabo raziskano (Uthes in sod., 2010). Uvajanje bolj ciljnih in rezultatsko usmerjenih ukrepov bo verjetno zahtevalo večjo prerazdelitev sredstev, ki se upravičencem dodeljujejo v okviru obstoječih KOP. Načrtovanje kmetijsko-okoljske politike v praksi ne temelji samo na doseganju okoljskih ciljev, temveč je odvisno tudi od bolj prikritih ciljev reševanja dohodkovnega vprašanja kmetijstva, ki je vzvod za ves javni intervencionizem na tem področju (Egdell, 2000; McCarthy in sod., 2018). Ti zmanjšujejo uspešnost kmetijsko-okoljskih ukrepov in tudi uporabo zahtevnejših mehanizmov podpore, kot so rezultatski ukrepi in skupno delovanje (Zilans in sod., 2019). Večje učinkovitosti in uresničevanja ciljev varstva okolja in narave zato ni mogoče doseči brez sprememb preferenc in vrednotenja okoljskih ciljev s strani neposrednih uporabnikov ukrepov – kmetijskih gospodarstev. Splošno razumevanje in sprejemanje okoljskih ciljev s strani kmetov, njihovih organizacij in celotnega kmetijskega institucionalnega sistema je zato po mnenju avtorjev nujen predpogoj za uveljavitev bolj ciljne in uspešnejše kmetijske-okoljske politike.

4.3 UPORABA IN PRIMERNOST RAZLIČNIH ZASNOV UKREPOV V SLOVENIJI

V okviru nekaterih projektov so v zadnjih letih v Sloveniji potekali pilotni poskusi mešanih in rezultatskih shem (Živikart in Debeljak, 2019). V raziskavi na dveh projektnih območjih v Sloveniji (Haloze in Kras) bi rezultatsko ali mešano zasnovo ukrepa za ekstenzivno rabo suhih travišč izbralo 78 % potencialnih upravičencev, obstoječa zasnova ukrepa Posebni traviščni habitati (HAB) pa je ustrezala preferencam zgolj vsakega sedmega (15 %) anketiranega kmeta (Šumrada in sod., 2020c). V prihodnjem programskem obdobju bi bilo zato verjetno smiselno preizkusiti nekatere ukrepe z mešano ali rezultatsko zasnovo, pri čemer bi bili ti verjetno najbolj primerni na področju ohranjanja naravovarstveno pomembnega travinja in določenih ogroženih vrst (Šumrada in sod., 2020c). Pri tem je v upravljanje ukrepa smiselno močneje vključiti tudi lokalne organizacije, kot so upravljavci zavarovanih območij, kmetijska svetovalna služba, lokalne akcijske skupine ter zainteresirane kmetijske in okoljske nevladne organizacije (O'Rourke in Finn, 2020; Rac in sod., 2020; Schomers in sod., 2015).

Na področju kmetijsko-okoljskih ukrepov v Sloveniji trenutno nimamo veliko izkušenj s koordiniranim in skupnim delovanjem upravičencev, čeprav v okviru nekaterih operacij lahko sodeluje več kmetijskih gospodarstev (npr. Planinska paša) (MKGP, 2017). Problem sodelovanja med kmetijskimi gospodarstvi na različnih področjih je bil v Sloveniji že večkrat izpostavljen (Erjavec in sod., 2018), vendar so razlogi za odklanjanje kmetov za sodelovanje, še posebej na področju sodelovanja v okoljskih ukrepih, slabo raziskani (Slovenc, 2019). Čeprav tovrstnih ukrepov zato na širših območjih še ni smotrno uvajati, pa potrebe po bolj koordiniranem pristopu k reševanju okoljskih problemov obstajajo in so bili kot izziv izpostavljeni tako s strani odločevalcev kot deležnikov (Erjavec in sod., 2018). Na manjših območjih bi bilo zato smiselno raziskati in preizkusiti pripravljenost za večje sodelovanje med akterji, kar bi v začetku lahko potekalo predvsem na ravni pilotskih projektov.

Slovenija je v preteklosti že poskušala izboljšati prostorsko ciljnost nekaterih kmetijsko-okoljskih ukrepov z opredelitvijo upravičenih območij in diferenciacijo plačil, in sicer predvsem na področju ohranjanja ekstenzivne rabe travinja (MKGP, 2017). Evalvacija izvajanja teh ukrepov kaže, da to ni bilo dovolj uspešno (Kaligarič in sod., 2019). V obdobju 2014–2020 so bile sicer pripravljene podrobnejše prostorske podlage, vendar prostorsko ciljanje ostaja šibkost obstoječih kmetijsko-okoljskih ukrepov v Sloveniji. To potrjuje tudi primerjalna analiza z drugimi državami članicami EU (ENRD, 2019). Ob upoštevanju pritiska po porabi sredstev kmetijske politike za druge namene bi bilo prostorsko ciljanje smiselno uvesti tudi pri ukrepih na drugih tipih rabe tal (njivske površine in trajni nasadi). S tem bi se verjetno izboljšala njihova rezultatska naravnanost. Na področju naravovarstvenih ukrepov je po drugi strani treba odpraviti neskladja med prostorskimi sloji, na katerih so opredeljene upravičene površine, in dejanskim stanjem v naravi (Kaligarič in sod., 2019). To je mogoče bodisi z izboljšanjem podatkovnih zbirk bodisi z razmislekom o alternativnih pristopih k določitvi površin.

Z izvajanjem izbora upravičencev z vsebinskim izborom med programi aktivnosti ali okoljskimi dražbami v okviru slovenske kmetijsko-okoljske politike v Sloveniji še nimamo izkušenj, pojavljajo se šele prve raziskave možnosti (Juvančič in sod., 2018). Gre za precej zahtevne tipe zasnov, ki verjetno presegajo trenutne potrebe in kapacitete. Poleg tega struktura kmetijstva z majhnimi in razdrobljenimi kmetijskimi gospodarstvi in s pretežno ostarelo in manj izobraženo delovno silo (Erjavec in sod., 2018) verjetno ne omogoča ustreznih razmer, da bi tovrstni ukrepi lahko dosegli kritično maso in domet uporabe (Grammatikopoulou in sod., 2012; Latacz-Lohmann in Schilizzi, 2005).

Zaključujemo z oceno, da v Sloveniji obstaja še precej možnosti za izboljšanje uspešnosti kmetijsko-okoljskih ukrepov (Kaligarič in sod., 2019; Slabe-Erker in sod., 2017; Šumrada in sod., 2020a, 2020b), vključno s smiselnimi nadgradnjami obstoječe zasnove shem s predpisanimi praksami. Podobno kot druge nove države članice EU tudi Slovenija na področju snovanja rezultatsko naravnanih in prostorsko ciljnih ukrepov trenutno še precej zaostaja v primerjavi s severnim in zahodnim delom Evrope (Herzon in sod., 2018). V okviru kmetijske politike je zato smiselno nadaljevati s pilotnimi poskusi in raziskavami, ki so se na projektni ravni pričeli v zadnjih letih. Eden od ključnih izzivov pri njihovem uvajanju pa bo tudi razvoj podpornega institucionalnega okolja, ki je močno odvisen od okrepitve zavedanja in sprejemljivosti vprašanj varstva narave in okolja pri vseh deležnikih, vključenih v slovensko kmetijsko politiko.

5 ZAHVALE

Prispevek je nastal v okviru Ciljnega raziskovalnega projekta (CRP V4-1814) Analitične podpore za večjo učinkovitost in ciljnost kmetijske politike do okolja in narave v Sloveniji in programa Ekonomika agroživilstva in naravnih virov (P4-0022) s finančno podporo Javne agencije za raziskovalno dejavnost in Ministrstva za kmetijstvo, gozdarstvo in prehrano.

6 VIRI

Allen, B., Hart, K., Radley, G., Tucker, G., Keenleyside, C., Oppermann, R., Underwood, E., Menadue, H., Poux, X., Beaufoy, G., Herzon, I., Povellato, A., Vanni, F., Pražan, J., Hudson, T., & Yellachich, N. (2014). *Biodiversity protection through results based remuneration of ecological achievement. Report Prepared for the European Commission, DG Environment, Contract No ENV.B.2/ETU/2013/0046* (str. 167). Institute for European Environmental Policy. https://ieep.eu/publications/results-based-agri-environment-schemes-new-report-and-guidance-handbook-available

Alliance Environment. (2019). *Evaluation of the impact of the CAP on habitats, landscapes, biodiversity.* Final Report. Evropska komisija, Generalni direktorat za kmetijstvo in razvoj podeželja. Pridobljeno s https://ec.europa.eu/info/sites/info/files/food-farming-fisheries/key_policies/documents/ext-eval-biodiversity-final-report_2020_en.pdf

Armsworth, P. R., Acs, S., Dallimer, M., Gaston, K. J., Hanley, N., & Wilson, P. (2012). The cost of policy simplification in conservation incentive programs. *Ecology Letters, 15*(5), 406–414. https://doi.org/10.1111/j.1461-0248.2012.01747.x

Batáry, P., Báldi, A., Kleijn, D., & Tscharntke, T. (2011). Landscape-moderated biodiversity effects of agri-environmental management: A meta-analysis. *Proceedings of the Royal Society B: Biological Sciences, 278*(1713), 1894–1902. https://doi.org/10.1098/rspb.2010.1923

Batáry, P., Dicks, L. V., Kleijn, D., & Sutherland, W. J. (2015). The role of agri-environment schemes in conservation and environmental management. *Conservation Biology, 29*(4), 1006–1016. https://doi.org/10.1111/cobi.12536

Berkhout, P., van Doorn, A., & Schrijver, R. (2018). *Targeted payments for services delivered by farmers: Possible approaches. Report 2018-052* (str. 38). Wageningen Economic Research. https://library.wur.nl/WebQuery/wurpubs/538061

Bertke, E., Klimek, S., & Wittig, B. (2008). Developing result-orientated payment schemes for environmental services in grasslands: Results from two case studies in North-western Germany. *Biodiversity,* 9(1–2), 91–95. https://doi.org/10.1080/14888386.2008.9712893

Birge, T., & Herzon, I. (2019). Exploring cultural acceptability of a hypothetical results-based agri-environment payment for grassland biodiversity. *Journal of Rural Studies, 67*, 1–11. https://doi.org/10.1016/j.jrurstud.2019.02.006

Birge, T., Toivonen, M., Kaljonen, M., & Herzon, I. (2017). Probing the grounds: Developing a payment-by-results agri-environment scheme in Finland. *Land Use Policy, 61*, 302–315. https://doi.org/10.1016/j.landusepol.2016.11.028

Boulton, A., Lockett, R., & Seymour, T. (2013). *A review and evaluation of collaborative landscape-scale management initiatives.* Commissioned Report no. 598. Scottish Natural Heritage. Pridobljeno s www.nls.uk/e-monographs/2013/598.pdf

Buller, H., Wilson, G. A., & Höll, A. (Ur.). (2000). *Agri-environmental Policy in the European Union.* Ashgate Publishing.

Burton, R. J. F., & Schwarz, G. (2013). Result-oriented agri-environmental schemes in Europe and their potential for promoting behavioural change. *Land Use Policy, 30*(1), 628–641. https://doi.org/10.1016/j.landusepol.2012.05.002

Chobotová, V. (2013). The role of market-based instruments for biodiversity conservation in Central and Eastern Europe. *Ecological Economics, 95*, 41–50. https://doi.org/10.1016/j.ecolecon.2013.08.007

Colombo, S., & Rocamora-Montiel, B. (2018). Result-oriented Agri-Environmental Climate Schemes as a means of promoting climate change mitigation in olive growing. *Outlook on Agriculture, 47*(2), 141–149. https://doi.org/10.1177/0030727018770931

de Lijster, E., & Prager, K. (2012). *The Use of Indicators in Agri-environmental Managemanent in the Netherlands. Indicators used by Dutch Agrarische Natuurverenigingen (ANVs) for Monitoring and Reporting their Activities* (str. 41). James Hutton Institute. https://www.researchgate.net/publication/283404773_The_Use_of_Indicators_in_Agri-environmental_Managemanent_in_the_Netherlands

ECA. (2011). *Is agri-environment support well designed and managed?.* Special report no. 7/2011. Evropsko računsko sodišče. Pridobljeno s https://www.eca.europa.eu/Lists/ECADocuments/SR11_07/SR11_07_EN.PDF

ECA. (2020). *Biodiversity on farmland: CAP contribution has not halted the decline.* Special report no. 13/2020. Evropsko računsko sodišče. Pridobljeno s https://www.eca.europa.eu/en/Pages/DocItem.aspx?did=53892

Egdell, J. (2000). Consultation on the countryside premium scheme: Creating a `market' for information. *Journal of Rural Studies, 16*(3), 357–366. https://doi.org/10.1016/S0743-0167(99)00039-X

ENRD. (2018). *Background briefing: Collective approaches.* Working Document of the Thematic Group (TG) on sustainable management of water and soils. European Network for Rural Development (ENRD). Pridobljeno s https://enrd.ec.europa.eu/sites/enrd/files/tg2_water-soil_briefing_collective-approaches.pdf

ENRD. (2019). *RDP analysis: Support to environment & climate change. M10.1 Agri-environment-climate commitments.* European Network for Rural Development (ENRD). Pridobljeno s https://enrd.ec.europa.eu/rdp-measures-analysis_en

Erjavec, E., Šumrada, T., Juvančič, L., Rac, I., Cunder, T., Bedrač, M., & Lovec, M. (2018). *Vrednotenje slovenske kmetijske politike v obdobju 2015-2020: Raziskovalna podpora za strateško načrtovanje po letu 2020.* Kmetijski inštitut Slovenije. Pridobljeno s https://www.researchgate.net/publication/329718206_Vrednotenje_slovenske_kmetijske_politike_v_obdobju_2015-2020_Raziskovalna_podpora_za_stratesko_nacrtovanje_po_letu_2020_-_Ljubljana_Kmetijski_institut_Slovenije

Evropska komisija. (2014). *Technical elements of agri-environment-climate measure in the programming period 2014-2020.* Evropska komisija.

Evropska komisija. (2018). Natural resources and environment—Legal texts and factsheets. Pridobljeno s https://ec.europa.eu/commission/publications/natural-resources-and-environment_en

FAO. (2011). *Payments for Ecosystem Services and Food Security.* Food and Agriculture Organization of the United Nations (FAO). Pridobljeno s http://www.fao.org/3/i2100e/i2100e00.htm

Fleury, P., Seres, C., Dobremez, L., Nettier, B., & Pauthenet, Y. (2015). "Flowering Meadows", a result-oriented agri-environmental measure: Technical and value changes in favour of biodiversity. *Land Use Policy,* 46, 103–114. https://doi.org/10.1016/j.landusepol.2015.02.007

Franks, J. R., & Emery, S. B. (2013). Incentivising collaborative conservation: Lessons from existing environmental Stewardship Scheme options. *Land Use Policy, 30*(1), 847–862. https://doi.org/10.1016/j.landusepol.2012.06.005

Gamero, A., Brotons, L., Brunner, A., Foppen, R., Fornasari, L., Gregory, R. D., … Voříšek, P. (2017). Tracking Progress Toward EU Biodiversity Strategy Targets: EU Policy Effects in Preserving its Common Farmland Birds. *Conservation Letters, 10*(4), 395–402. https://doi.org/10.1111/conl.12292

Gerowitt, B., Isselstein, J., & Marggraf, R. (2003). Rewards for ecological goods—Requirements and perspectives for agricultural land use. *Biotic Indicators for Biodiversity and Sustainable Agriculture, 98*(1), 541–547. https://doi.org/10.1016/S0167-8809(03)00112-9

Gorton, M., Hubbard, C., & Hubbard, L. (2009). The Folly of European Union Policy Transfer: Why the Common Agricultural Policy (CAP) Does Not Fit Central and Eastern Europe. *Regional Studies, 43*(10), 1305–1317. https://doi.org/10.1080/00343400802508802

Grammatikopoulou, I., Iho, A., & Pouta, E. (2012). Willingness of farmers to participate in agri-environmental auctions in Finland. *Food Economics, 9*(4), 215–230. https://doi.org/10.1080/2164828X.2013.845557

Groth, M. (2009). *The transferability and performance of payment-by-results biodiversity conservation procurement auctions: Empirical evidence from northernmost Germany* (št. 119; Working Paper Series in Economics, str. 35). https://econpapers.repec.org/paper/luewpaper/119.htm

Heinz, S., Mayer, F., & Kuhn, G. (2018). *Artenreiches Grünland: Ergebnisorientierte Grünlandnutzung. Artenliste & Bestimmungshilfe.* Bayerische Landesanstalt für Landwirtschaft (LfL). https://www.lfl.bayern.de/publikationen/informationen/069544/index.php

Herzon, I., Birge, T., Allen, B., Povellato, A., Vanni, F., Hart, K., … Pražan, J. (2018). Time to look for evidence: Results-based approach to biodiversity conservation on farmland in Europe. *Land Use Policy, 71,* 347–354. https://doi.org/10.1016/j.landusepol.2017.12.011

Huige, R., R. Lapperre, in G. Stanton. (2010). „The WTO context". V A. Oskam, G. Meester, & H. Silvis (Ur.), *EU Policy for Agriculture, Food and Rural Areas* (str. 89–101). Wageningen Academic Publishers.

Iho, A., Lankoski, J., Ollikainen, M., Puustinen, M., & Lehtimäki, J. (2014). Agri-environmental auctions for phosphorus load reduction: Experiences from a Finnish pilot. *Australian Journal of Agricultural and Resource Economics, 58*(2), 205–222. https://doi.org/10.1111/1467-8489.12049

Juvančič, L., Bojkovski, D., Pohar, J., Kuhar, A., & Vrisk, M. (2018). *Določitev stroškovno učinkovitih javnih podpor za ohranjanje avtohtonih pasem domačih živali V4-1433.* Končno poročilo o rezultatih opravljenega raziskovalnega dela na projektu v okviru ciljnega raziskovalnega programa (CRP) »Zagotovimo.si hrano za jutri«. Univerza v Ljubljani, Biotehniška fakulteta. Pridobljeno s http://www.dlib.si/?URN=URN:NBN:SI:DOC-WHEX7470

Kaiser, T., Reutter, M., & Matzdorf, B. (2019). How to improve the conservation of species-rich grasslands with result-oriented payment schemes? *Journal for Nature Conservation, 52*, 125752. https://doi.org/10.1016/j.jnc.2019.125752

Kaiser, T., Rohner, M., Matzdorf, B., & Kiesel, J. (2010). Validation of grassland indicator species selected for result-oriented agri-environmental schemes. *Biodiversity and Conservation,* 19(5), 1297–1314. https://doi.org/10.1007/s10531-009-9762-8

Kaligarič, M., Čuš, J., Škornik, S., & Ivajnšič, D. (2019). The failure of agri-environment measures to promote and conserve grassland biodiversity in Slovenia. *Land Use Policy, 80,* 127–134. https://doi.org/10.1016/j.landusepol.2018.10.013

Keenleyside, C., Allen, B., Hart, K., Menadue, H., Stefanova, V., Prazan, J., Herzon, I., Clement, T., Povellato, A., Maciejczak, M., & Boatman, N. (2011). *Delivering environmental benefits through entry level agri-environment schemes in the EU. Report prepared for DG Environment, Project ENV.B.1/ETU/2010/0035* (str. 209). Institute for European Environmental Policy. https://ieep.eu/publications/delivering-environmental-benefits-through-entry-level-agri-environment-schemes-in-the-eu

Kleijn, D., & Sutherland, W. J. (2003). How effective are European agri-environment schemes in conserving and promoting biodiversity? *Journal of Applied Ecology, 40*(6), 947–969. https://doi.org/10.1111/j.1365-2664.2003.00868.x

Krall, A., & Toth, P. (2015). *A Magas Termeszeti Ertekű Területek tamogatasi lehetőségei: Tajekoztato füzet gazdalkodoknak 2015.* Magyar Madártani és Természetvédelmi Egyesület (MME). http://natura.2000.hu/hu/filedepot_download/636/776

Krämer, J. E., & Wätzold, F. (2018). The agglomeration bonus in practice—An exploratory assessment of the Swiss network bonus. *Journal for Nature Conservation, 43,* 126–135. https://doi.org/10.1016/j.jnc.2018.03.002

Lastra-Bravo, X. B., Hubbard, C., Garrod, G., & Tolón-Becerra, A. (2015). What drives farmers' participation in EU agri-environmental schemes?: Results from a qualitative meta-analysis. *Environmental Science & Policy, 54*, 1–9. https://doi.org/10.1016/j.envsci.2015.06.002

Latacz-Lohmann, U., & Schilizzi, S. (2005). *Auctions for Conservation Contracts: A Review of the Theoretical and Empirical Literature* (Project No: UKL/001/05). University of Kiel; University of Western Australia. Pridobljeno s https://www2.gov.scot/Publications/2006/02/21152441/0

Leventon, J., Schaal, T., Velten, S., Dänhardt, J., Fischer, J., Abson, D. J., & Newig, J. (2017). Collaboration or fragmentation? Biodiversity management through the common agricultural policy. *Land Use Policy, 64,* 1–12. https://doi.org/10.1016/j.landusepol.2017.02.009

Lundberg, L., Persson, U. M., Alpizar, F., & Lindgren, K. (2018). Context Matters: Exploring the Cost-effectiveness of Fixed Payments and Procurement Auctions for PES. *Ecological Economics, 146,* 347–358. https://doi.org/10.1016/j.ecolecon.2017.11.021

Matzdorf, B., Kaiser, T., & Rohner, M. (2008). Developing biodiversity indicator to design efficient agri-environmental schemes for extensively used grassland. *Ecological Indicators,* 8(3), 256–269. https://doi.org/10.1016/j.ecolind.2007.02.002

McCarthy, J., Bonnin, C., & Meredith, D. (2018). Disciplining the State: The role of alliances in contesting multi-level agri-environmental governance. *Land Use Policy, 76,* 317–328. https://doi.org/10.1016/j.landusepol.2018.04.049

McKenzie, A. J., Emery, S. B., Franks, J. R., & Whittingham, M. J. (2013). Landscape-scale conservation: Collaborative agri-environment schemes could benefit both biodiversity and ecosystem services, but will farmers be willing to participate? *Journal of Applied Ecology, 50*(5), 1274–1280. https://doi.org/10.1111/1365-2664.12122

Meyer, C., Reutter, M., Matzdorf, B., Sattler, C., & Schomers, S. (2015). Design rules for successful governmental payments for ecosystem services: Taking agri-environmental measures in Germany as an example. *Journal of Environmental Management, 157,* 146–159. https://doi.org/10.1016/j.jenvman.2015.03.053

Mills, J., Gibbon, D., Ingram, J., Reed, M., Short, C., & Dwyer, J. (2011). Organising Collective Action for Effective Environmental Management and Social Learning in Wales. *The Journal of Agricultural Education and Extension, 17*(1), 69–83. https://doi.org/10.1080/1389224X.2011.536356

Ministry of Economic Affairs. (2016). *The cooperative approach under the new Dutch agri-environment-climate scheme: Background, procedures and legal and institutional implications.* Ministry of Economic Affairs. Pridobljeno s https://enrd.ec.europa.eu/sites/enrd/files/w12_collective-approach_nl.pdf

MKGP. (2017). Program razvoja podeželja RS za obdobje 2014-2020 (CCI 2014SI06RDNP001). Različica 5.1. Evropska komisija. Pridobljeno s https://www.program-podezelja.si/images/SPLETNA_STRAN_PRP_NOVA/1_PRP_2014-2020/1_1_Kaj_je_program_razvoja_pode%C5%BEelja/3._sprememba_PRP/Tretja_sprememba_PRP_Programme_2014SI06RDNP001_5_1_sl.pdf

Natural England. (2017). *Guide to Countryside Stewardship: Facilitation fund 2017.* Natural England. https://www.gov.uk/government/publications/guide-to-countryside-stewardship-facilitation-fund

Newton, I. (2017). *Farming and Birds.* HarperCollins.

OECD. (2010). *Guidelines for Cost-effective Agri-environmental Policy Measures.* OECD. https://doi.org/10.1787/9789264086845-en

OECD. (2013). *Providing Agri-environmental Public Goods through Collective Action.* OECD. https://doi.org/10.1787/9789264197213-en

OECD. (2015). *Public Goods and Externalities: Agri-environmental Policy Measures in Selected OECD Countries.* OECD. https://doi.org/10.1787/9789264239821-en

OECD. (2017). *Evaluation of Agricultural Policy Reforms in the*

European Union: The Common Agricultural Policy 2014-20. OECD. https://doi.org/10.1787/9789264278783-en

Oranmore. (2019). *Hen Harrier Programme: Terms and Conditions.* Hen Harrier Project. http://www.henharrierproject.ie/resources.html#

O'Rourke, E., & Finn, J. A. (Ur.). (2020). *Farming for Nature: The role of results-based payments. Teagasc*; National Parks and Wildlife Service. Pridobljeno s https://www.teagasc.ie/environment/biodiversity--countryside/farming-for-nature/

Palm-Forster, L. H., Swinton, S. M., Lupi, F., & Shupp, R. S. (2016). Too Burdensome to Bid: Transaction Costs and Pay-for-Performance Conservation. *American Journal of Agricultural Economics, 98*(5), 1314–1333. https://doi.org/10.1093/ajae/aaw071

Parkhurst, G. M., & Shogren, J. F. (2007). Spatial incentives to coordinate contiguous habitat. *Special Section - Ecosystem Services and Agriculture, 64*(2), 344–355. https://doi.org/10.1016/j.ecolecon.2007.07.009

PEGASUS. (2018). Case studies. *Public Ecosystem Goods and Services from land management - Unlocking the Synergies (PEGASUS).* http://pegasus.ieep.eu/case-studies/introduction

Perrot-Maître, D. (2006). *The Vittel payments for ecosystem services: A "perfect" PES case?* (str. 24). International Institute for Environment and Development. https://pubs.iied.org/G00388/

Plieninger, T., Schleyer, C., Schaich, H., Ohnesorge, B., Gerdes, H., Hernández-Morcillo, M., & Bieling, C. (2012). Mainstreaming ecosystem services through reformed European agricultural policies. *Conservation Letters, 5*(4), 281–288. https://doi.org/10.1111/j.1755-263X.2012.00240.x

Poláková, J., Tucker, G., Hart, K., Dwyer, J., & Rayment, M. (2011). Addressing biodiversity and habitat preservation through Measures applied under the Common Agricultural Policy. Report Prepared for DG Agriculture and Rural Development, Contract No. 30-CE-0388497/00-44 (str. 357). Institute for European Environmental Policy. Pridobljeno s https://ieep.eu/publications/addressing-the-eu-s-biodiversity-goals-through-the-cap

Polman, N., Slangen, L., & Huylenbroeck, G. (2010). Collective approaches to agri-environmental management. V *EU Policy for Agriculture, Food and Rural Areas* (str. 363–368). Wageningen Academic Publishers.

Prager, K. (2015). Agri-environmental collaboratives for landscape management in Europe. *Sustainability governance and transformation, 12*, 59–66. https://doi.org/10.1016/j.cosust.2014.10.009

Rac, I., Juvančič, L., & Erjavec, E. (2020). Stimulating collective action to preserve High Nature Value farming in post-transitional settings. A comparative analysis of three Slovenian social-ecological systems. *Nature Conservation, 39,* 87–111. https://doi.org/10.3897/natureconservation.39.51216

RBAPS. (2020). Inventory of results-based agri-environment schemes. *Farming for Biodiversity: The results-based agri-environment schemes.* http://ec.europa.eu/environment/nature/rbaps/fiche/search/index_en.htm

Reed, M. S., Moxey, A., Prager, K., Hanley, N., Skates, J., Bonn, A., … Thomson, K. (2014). Improving the link between payments and the provision of ecosystem services in agri-environment schemes. *Ecosystem Services, 9,* 44–53. https://doi.org/10.1016/j.ecoser.2014.06.008

Riley, M., Sangster, H., Smith, H., Chiverrell, R., & Boyle, J. (2018). Will farmers work together for conservation? The potential limits of farmers' cooperation in agri-environment measures. *Land Use Policy, 70,* 635–646. https://doi.org/10.1016/j.landusepol.2017.10.049

Rolfe, J., Whitten, S., & Windle, J. (2017). The Australian experience in using tenders for conservation. *Land Use Policy, 63,* 611–620. https://doi.org/10.1016/j.landusepol.2015.01.037

Ruiz-Mirazo, J., Robles, A. B., & González-Rebollar, J. L. (2011). Two-year evaluation of fuelbreaks grazed by livestock in the wildfire prevention program in Andalusia (Spain). *Agriculture, Ecosystems & Environment,* 141(1), 13–22. https://doi.org/10.1016/j.agee.2011.02.002

Russi, D., Margue, H., & Keenleyside, C. (2014). *Result-Based Agri-Environment Measures: Market-Based Instruments, Incentives or Rewards? The case of Baden-Württemberg. A case-study report prepared by IEEP with funding from the Invaluable project* (str. 44). Institute for European Environment al Policy. https://ieep.eu/publications/result-based-agri-environment-measures-market-based-instruments-incentives-or-rewards

Saba, A. (2017). Results-Based Agri-Environmental Schemes for Delivering Ecosystem Services in the EU: Established Issues and Emerging Trends. V M. Alabrese, M. Brunori, S. Rolandi, & A. Saba (Ur.), *Agricultural Law* (str. 83–122). Springer International Publishing. https://doi.org/10.1007/978-3-319-64756-2_5

Sabatier, R., Doyen, L., & Tichit, M. (2012). Action versus Result-Oriented Schemes in a Grassland Agroecosystem: A Dynamic Modelling Approach. *PLoS ONE, 7*(4), e33257. https://doi.org/10.1371/journal.pone.0033257

Schilizzi, S. (2017). An overview of laboratory research on conservation auctions. *Land Use Policy, 63,* 572–583. https://doi.org/10.1016/j.landusepol.2015.06.035

Schomers, S., Matzdorf, B., Meyer, C., & Sattler, C. (2015). How Local Intermediaries Improve the Effectiveness of Public Payment for Ecosystem Services Programs: The Role of Networks and Agri-Environmental Assistance. *Sustainability, 7*(10), 13856–13886. https://doi.org/10.3390/su71013856

Schwarz, G., Moxey, A., McCracken, D., Huband, S., & Cummins, R. (2008). *An analysis of the potential effectiveness of a Payment-by-Results approach to the delivery of environmental public goods and services supplied by Agri-Environment Schemes. Report to the Land Use Policy Group* (str. 92). Macaulay Institute, Pareto Consulting and Scottish Agricultural College. http://publications.naturalengland.org.uk/publication/5275491043901440

Sidemo-Holm, W., Smith, H. G., & Brady, M. V. (2018). Improving agricultural pollution abatement through result-based payment schemes. *Land Use Policy, 77,* 209–219. https://doi.org/10.1016/j.landusepol.2018.05.017

Slabe-Erker, R., Bartolj, T., Ogorevc, M., Kavaš, D., & Koman, K. (2017). The impacts of agricultural payments on groundwater quality: Spatial analysis on the case of Slovenia. *Eco-*

logical Indicators, 73, 338–344. https://doi.org/10.1016/j.ecolind.2016.09.048

Slovenc, M. (2019). Can a "Good Farmer" and a "Bad Farmer" Cooperate?: An Examination of Conventional and Organic Farmers' Perceptions of Production and Environmental Protection. V A. A. Lukšič & T. Tkalec (Ur.), *Intertwining of diverse minds in(to) political ecology: Scientific texts of doctoral students participating in the Summer school of political ecology* (str. 111–129). Inštitut Časopis za kritiko znanosti. Pridobljeno s https://www.researchgate.net/publication/340681391_Can_a_Good_Farmer_and_a_Bad_Farmer_Cooperate_An_Examination_of_Conventional_and_Organic_Farmers'_Perceptions_of_Production_and_Environmental_Protection

Stolze, M., Frick, R., Schmid, O., Stöckli, S., Bogner, D., Chevillat, V., Dubbert, M., Fleury, P., Neuner, S., Nitsch, H., Plaikner, M., Schramek, J., Tasser, E., Vincent, A., & Wezel, A. (2015). *Result-oriented Measures for Biodiversity in Mountain Farming. A Policy Handbook.* Research Institute of Organic Agriculture (FiBL). http://orgprints.org/32259/1/merit-policy-handbook-english-download.pdf

Sutcliffe, L. M. E., Batáry, P., Kormann, U., Báldi, A., Dicks, L. V., Herzon, I., … Tscharntke, T. (2015). Harnessing the biodiversity value of Central and Eastern European farmland. *Diversity and Distributions, 21*(6), 722–730. https://doi.org/10.1111/ddi.12288

Šumrada, T., Kmecl, P., & Erjavec, E. (2020a). Učinki kmetijske rabe in ukrepov javnih politik na pestrost ptic kmetijske krajine. V J. Prišenk (Ur.), *Razvojni vidiki prenosa znanja v skupni kmetijski politiki po letu 2020* (str. 41–53). Društvo agrarnih ekonomistov Slovenije (DAES). Pridobljeno s http://www.daes.si/Splet/8.%20konferenca%20DAES%20-%20Zbornik.pdf

Šumrada, T., Lovec, M., Juvančič, L., Rac, I., & Erjavec, E. (2020b). Fit for the task? Integration of biodiversity policy into the post-2020 Common Agricultural Policy: Illustration on the case of Slovenia. *Journal for Nature Conservation, 54,* 125804. https://doi.org/10.1016/j.jnc.2020.125804

Šumrada, T., Novak, A., Udovč, A., Rac, I., Šilc, U., Čelik, T., … Erjavec, E. (2020c). *Potenciali novih zasnov in sodelovanje kmetov v kmetijsko-okoljskih ukrepih.* Poročilo v okviru CRP V4-1814 Analitične podpore za večjo učinkovitost in ciljnost kmetijske politike do okolja in narave v Sloveniji. Univerza v Ljubljani, Biotehniška fakulteta.

Uhl, H. (2018). *Zwischenbericht zum Artenschutzprojekt Heidelerche im Mühlviertel 2018* (str. 11). BirdLife Österreich.

Uhl, H., & Wichmann, G. (2017). *Artenschutz- und Monitoring-Projekte zugunsten gefährdeter Kulturlandschaftsvögel in Oberösterreich 2015-2017. Projektbericht* (str. 86). BirdLife Österreich. https://www.birdlife.at/web/binary/saveas?filename_field=datas_fname&field=datas&model=ir.attachment&id=8167

Uthes, S., & Matzdorf, B. (2013). Studies on Agri-environmental Measures: A Survey of the Literature. *Environmental Management, 51*(1), 251–266. https://doi.org/10.1007/s00267-012-9959-6

Uthes, S., Matzdorf, B., Müller, K., & Kaechele, H. (2010). Spatial Targeting of Agri-Environmental Measures: Cost-Effectiveness and Distributional Consequences. *Environmental Management, 46*(3), 494–509. https://doi.org/10.1007/s00267-010-9518-y

Vainio, A., Tienhaara, A., Haltia, E., Hyvönen, T., Pyysiäinen, J., & Pouta, E. (2019). The legitimacy of result-oriented and action-oriented agri-environmental schemes: A comparison of farmers' and citizens' perceptions. *Land Use Policy,* 104358. https://doi.org/10.1016/j.landusepol.2019.104358

Verhulst, J. (2007). *Meadow bird ecology at different spatial scales—Responses to environmental conditions and implications for management* [doktorska disertacija, Wageningen University]. https://edepot.wur.nl/2489

Vesterager, J. P., Frederiksen, P., Kristensen, S. B. P., Vadineanu, A., Gaube, V., Geamana, N. A., … Busck, A. G. (2016). Dynamics in national agri-environmental policy implementation under changing EU policy priorities: Does one size fit all? *Land Use Policy, 57,* 764–776. https://doi.org/10.1016/j.landusepol.2015.05.014

Vojtech, V. (2010). *Policy Measures Addressing Agri-environmental Issues* (OECD Food, Agriculture and Fisheries Papers no. 24). OECD. https://doi.org/10.1787/5kmjrzg08vvb-en

Wätzold, F., & Drechsler, M. (2005). Spatially Uniform versus Spatially Heterogeneous Compensation Payments for Biodiversity-Enhancing Land-Use Measures. *Environmental & Resource Economics, 31*(1), 73–93. https://doi.org/10.1007/s10640-004-6979-6

Wätzold, F., & Drechsler, M. (2014). Agglomeration payment, agglomeration bonus or homogeneous payment? *Resource and Energy Economics, 37,* 85–101. https://doi.org/10.1016/j.reseneeco.2013.11.011

Westerink, J., Jongeneel, R., Polman, N., Prager, K., Franks, J. R., Dupraz, P., & Mettepenningen, E. (2017). Collaborative governance arrangements to deliver spatially coordinated agri-environmental management. *Land Use Policy, 69,* 176–192. https://doi.org/10.1016/j.landusepol.2017.09.002

Wezel, A., Vincent, A., Nitsch, H., Schmid, O., Dubbert, M., Tasser, E., … Bogner, D. (2018). Farmers' perceptions, preferences, and propositions for result-oriented measures in mountain farming. *Land Use Policy, 70,* 117–127. https://doi.org/10.1016/j.landusepol.2017.10.020

Wezel, A., Zipfer, M., Aubry, C., Barataud, F., & Heißenhuber, A. (2016). Result-oriented approaches to the management of drinking water catchments in agricultural landscapes. *Journal of Environmental Planning and Management, 59*(2), 183–202. https://doi.org/10.1080/09640568.2014.1000453

Wittig, B., Richter gen. Kemmermann, A., & Zacharias, D. (2006). An indicator species approach for result-orientated subsidies of ecological services in grasslands – A study in Northwestern Germany. *Biological Conservation,* 133(2), 186–197. https://doi.org/10.1016/j.biocon.2006.06.004

Zabel, A. (2019). Biodiversity-based payments on Swiss alpine pastures. *Land Use Policy,* 81, 153–159. https://doi.org/10.1016/j.landusepol.2018.10.035

Zabel, A., & Holm-Müller, K. (2008). Conservation Performance Payments for Carnivore Conservation in Sweden. *Conservation Biology,* 22(2), 247–251. https://doi.org/10.1111/j.1523-1739.2008.00898.x

Zavalloni, M., Raggi, M., & Viaggi, D. (2019). Agri-environmental Policies and Public Goods: An Assessmentof Coalition Incentives and Minimum Participation Rules. *Environmental and Resource Economics, 72*(4), 1023–1040. https://doi.org/10.1007/s10640-018-0237-9

Zilans, A., Schwarz, G., Veidemane, K., Osbeck, M., Tonderski, A., & Olsson, O. (2019). Enabling policy innovations promoting multiple ecosystem benefits: Lessons learnt from case studies in the Baltic Sea Region. *Water Policy, 21*(3), 546–564. https://doi.org/10.2166/wp.2019.054

Žvikart, M., & Debeljak, N. (2019). Ovrednotenje stroškov priprave in izvajanja rezultatsko usmerjenega kmetijsko-okoljskega ukrepa za ohranjanje suhih travišč. *Varstvo narave, 31,* 47–60.

Priloga: Pregled primerov zasnov kmetijsko-okoljskih ukrepov v evropskih državah glede na tri področja načrtovanja ukrepov

Naziv ukrepa in izvorno ime	Država	Regija	Obdobje	Cilji sheme	Osnova za plačilo	Sklepanje pogodb	Izbor upravičencev	Viri
Rezultatski naravovarstveni načrt *(Ergebnisorientierter Naturschutzplan, ENP)*	Avstrija		2014-danes	Narava	Rezultatska	Individualno	Splošna	RBAPS (2020), Allen idr. (2014)
Varstveni projekt za hribskega škrjanca v narodnem parku Mühlviertel	Avstrija	Gornja Avstrija	2015?-danes	Narava	Mešana	Posrednik	Prostorsko ciljanje	Uhl in Wichmann (2017), Uhl (2018)
Vzpostavitev vodovarstvenih pasov v dolini Dommel	Belgija	Limburg	2006-2013?	Vode	Predpisane prakse	Posrednik	Prostorsko ciljanje	OECD (2013)
Upravljanje s kakovostjo voda s strani vodnega podjetja Pidpa in kmetov	Belgija	Antwerp	?	Vode	Predpisane prakse	Oddaja v upravljanje	Prostorsko ciljanje	OECD (2013)
Kmetijsko-okoljski ukrep za preprečevanje erozije tal	Češka		2004-danes	Narava; Okolje	Predpisane prakse	Individualno	Prostorsko ciljanje	Berkhout idr. (2018), Keenleyside idr. (2011)
Varstvena shema za planinskega orla	Finska	skupnost Sami	1998-danes?	Narava	Rezultatska	Skupna organizacija	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014)
Načrt upravljanja s pašniki *(Gestion pastorale: HERBE_09)*	Francija		2007-danes?	Narava; Požari	Mešana	Individualno	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014)
Ohranjanje vrstno bogatih travišč *(Prairies Fleuries: HERBE_07)*	Francija		2007-danes	Narava	Mešana	Individualno	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014), Fleury idr. (2015)
Pogodbeno varstvo med podjetjem Vittel in kmeti	Francija	Vosges	1988-danes	Vode	Predpisane prakse	Posrednik in oddaja v najem	Prostorsko ciljanje	OECD (2013), Perrot-Maitre (2006)
Varstveni program za območje Burren	Irska	Clare	2010-danes	Narava; Krajina	Mešana	Posrednik	Prostorsko ciljanje	O'Rourke in Finn (2020), RBAPS (2020), Allen idr. (2014)
Varstveni program za območje Aranskih otokov	Irska	Galway	2015-danes	Narava; Krajina	Krajina	Posrednik	Prostorsko ciljanje	O'Rourke in Finn (2020)
Program za varstvo pepelastega lunja *(Hen Harrier Programme)*	Irska		2015-danes	Narava	Mešana	Posrednik	Vsebinski izbor	Oranmore (2019), O'Rourke in Finn (2020)
Shema službe za zavarovana območja in varstvo narave *(NPWS Farm Plan Scheme)*	Irska		2006-danes	Narava	Predpisane prakse	Posrednik	Prostorsko ciljanje	O'Rourke in Finn (2020)
Kmetijsko-okoljski program v Valdasi	Italija	Marke	2009-danes	Voda; Tla	Predpisane prakse	Posrednik	Prostorsko ciljanje	ENRD (2018), PEGASUS (2018)
Ukrepi za izboljšanje poplavne varnosti v Toskani	Italija	Toskana	?	Poplave	Predpisane prakse	Posrednik	Prostorsko ciljanje	OECD (2013)
Ukrepi na območjih z visoko naravno vrednostjo *(MTÉT)*	Madžarska		2002-danes	Narava	Predpisane prakse	Posrednik	Prostorsko ciljanje	Krall in Toth (2015)
Ohranjanje vrstno bogatih travišč *(MEKA-B4, FAKT-B3)*	Nemčija	Baden-Württemberg	2000-danes	Narava	Mešana	Individualno	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014), Russi idr. (2014)
Ohranjanje vrstno bogatih travišč *(PAULa)*	Nemčija	Rheinland-Pfalz	2007-danes?	Narava	Mešana	Individualno	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014)

Nadaljevanje

Ohranjanje vrstno bogatih travišč	Nemčija	Niedersachsen in Bremen	2007-danes?	Narava	Mešana	Individualno	Prostorsko ciljanje	Allen idr. (2014), Bertke idr. (2008), Wittig idr. (2006)
Ohranjanje vrstno bogatih travišč	Nemčija	Brandenburg	2008-danes?	Narava	Mešana	Individualno	Prostorsko ciljanje	Allen idr. (2014), Kaiser idr. (2010), Matzdorf idr. (2008)
Program Blühendes Steinburg	Nemčija	Schleswig-Holstein	2007-?	Narava	Rezultatska	Individualno	Okoljska dražba	RBAPS (2020), Groth (2009)
Varstveni ukrepi za travniške vrste ptic	Nemčija	Schleswig-Holstein	1997-danes?	Narava	Mešana	Posrednik	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014)
Ukrepi za zaščito gnezd lunjev na njivskih površinah	Nemčija	Nordrhein-Westfalen	1993-danes?	Narava	Mešana	Posrednik	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014)
Ohranjanje tradicionalnih sadovnjakov	Nemčija		2007-danes?	Krajina	Mešana	Individualno	Prostorsko ciljanje	RBAPS (2020)
Ohranjanje vrstno bogatih travišč *(B40)*	Nemčija	Bavarska	2015-danes	Narava	Rezultatska	Individualno	Prostorsko ciljanje	Stolze idr. (2015), Heinz idr. (2018)
Ustanova Rheinische Kulturlandschaft	Nemčija	Rheinland	2003-danes	Narava; Krajina	Predpisane prakse	Oddaja v upravljanje	Prostorsko ciljanje	Westerink in sod. (2017)
Obnovitev mokrišč v dolini Eider	Nemčija	Schleswig-Holstein	1999-danes?	Podnebje; Narava; Voda	Predpisane prakse	Oddaja v upravljanje	Prostorsko ciljanje	OECD (2013)
Program za zaščito in izboljšanje kakovosti podzemnih voda v mestu Augsburg	Nemčija		1988-danes	Voda	Mešana	Individualno	Prostorsko ciljanje	Wezel idr. (2016)
Sodelovanje na področju varstva pitne vode	Nemčija	Spodnja Saška	1992-danes?	Voda	Predpisane prakse	Skupna organizacija	Prostorsko ciljanje	OECD (2013)
Pilotna shema za varstvo gnezdečih travniških vrst ptic (Univerza v Leidnu)	Nizozemska		1993-1996	Narava	Rezultatska	Individualno	Prostorsko ciljanje	RBAPS (2020), Schwarz idr. (2008)
Ohranjanje vrstno bogatih travišč in njivskih vrst rastlin	Nizozemska		2000-2006	Narava	Mešana	Individualno	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014), Schwarz idr. (2008)
Varstveni ukrepi za travniške vrste ptic	Nizozemska		2000-2004	Narava	Mešana	Skupna organizacija	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014), Verhulst idr. (2007)
Varstveni ukrepi za travniške vrste ptic v okviru kmetijsko-okoljskih skupnosti	Nizozemska		2005-2015	Narava	Rezultatska	Skupna organizacija	Prostorsko ciljanje	RBAPS (2020), OECD (2013), Allen idr. (2014), de Lijster & Prager (2012)
Kmetijsko-okoljske skupnosti	Nizozemska		2015-danes	Okolje; Narava	Različno	Skupna organizacija	Prostorsko ciljanje	Westerink in sod. (2017)
Omrežje pašnih in protipožarnih območij v Andaluziji *(RAPCA)*	Španija	Andaluzija	2005-danes?	Požari	Rezultatska	Posrednik	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014), Ruiz-Mirazo idr. (2011)
Varstvena plačila za ohranjanje risa in rosomaha	Švedska	skupnost Sami	1996-danes	Narava	Rezultatska	Skupna organizacija	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014), Zabel in Holm-Müller (2008)

Nadaljevanje

Pašna skupnost Söne Mad	Švedska	Västergötland	1995-danes?	Narava; Krajina	Predpisane prakse	Skupna organizacija	Prostorsko ciljanje	OECD (2013)
Ohranjanje vrstno bogatih travišč in vinogradov *(ÖQV)*	Švica		1995-danes?	Narava	Mešana	Individualno	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014)
Skupinski bonus *(ÖQV-Vernetzungsprojekte)*	Švica		2001-danes	Narava	Predpisane prakse	Skupinski bonus	Prostorsko ciljanje	Krämer in Wätzold (2018)
Plačila za ohranjanje biodiverzitete na planinskih pašnikih	Švica		2014-danes	Narava	Rezultatska	Individualno ali skupna organizacija	Prostorsko ciljanje	Zabel (2019)
Kmetijska naravovarstvena shema v narodni park Peak District	Velika Britanija	Anglija	1987-1997	Narava; Krajina	Mešana	Individualno	Prostorsko ciljanje	RBAPS (2020), Allen idr. (2014), Schwarz idr. (2008)
Podpora za skupninske prijave (HR8, Higher level Environmental Stewardship Scheme)	Velika Britanija	Anglija	1994-2015	Narava; Krajina	Predpisane prakse	Skupna organizacija	Prostorsko ciljanje	Westerink in sod. (2017)
Podporni ukrep za skupinske prijave *(Countryside Stewardship facilitation fund)*	Velika Britanija	Anglija	2015-danes		Mešana	Skupna organizacija	Okoljska dražba	ENRD (2018), Natural England (2017)

doi:10.14720/aas.2020.116.1.1477

An updated data on the bionomics of pomegranate fruit borer, *Deudorix epijarbas* (Moore, 1858) (Lepidoptera: Lycaenidae), infesting pomegranates in Kashmir

Insha YOUSUF [1, 2] and Abdul A. BUHROO [1]

Received February 01, 2020; accepted September 01, 2020.
Delo je prispelo 01. februarja 2020, sprejeto 01. septembra 2020.

An updated data on the bionomics of pomegranate fruit borer, *Deudorix epijarbas* (Moore, 1858) (Lepidoptera: Lycaenidae), infesting pomegranates in Kashmir

Abstract: Pomegranate fruit borer, *Deudorix epijarbas* (Moore, 1858) (Lepidoptera: Lycaenidae) is a destructive pest of pomegranate affecting the quality as well as quantity of its fruits. The pest and its other allied species are nuisance to pomegranate cultivation throughout India, particularly in hilly state of Jammu & Kashmir. The aim of present investigation was to study the bionomics of pomegranate fruit borer in laboratory, which included the studies on its morphometry and extent of different developmental stages in the life cycle of pest, for developing a sound management plan against it. The results revealed that the eggs are laid by females singly on fruits, stalks, leaves and flower buds. On hatching, emerging larva bores in to fruits and feed on its pulp thus rendering them unfit for trade. The pest passed through five larval instars of different duration and the total larval period was 32.20 ± 1.87 (SD) days. The larval stage was followed by pupal stage which lasted for 9.50 ± 1.84 (SD) days. Furthermore, the longevity of adult male and female butterflies was recorded as 9.20 ± 1.73 (SD) and 13.20 ± 1.43 (SD) days respectively. The study thus provides information necessary for development and implementation of different control strategies in IPM of pomegranate fruit borer.

Key words: pomegranate; *Deudorix epijarbas*; bionomics; morphometry; larval instars

Dopolnjeni podatki o bionomiki zavijača granatnega jabolka *Deudorix epijarbas* (Moore, 1858) (Lepidoptera: Lycaenidae) v Kašmirju

Izvleček: Zavijač granatnega jabolka, *Deudorix epijarbas* (Moore, 1858) (Lepidoptera: Lycaenidae) je uničujoč škodljivec, ki vpliva na kakovost in količino plodov. Ta in sorodne vrste škodljivcev predstavljajo težave pri gojenju granatnega jabolka širom Indije, še posebej v hriboviti državi Jammu & Kashmir. Namen raziskave je bil preučiti bionomiko zavijača granatnega jabolka v laboratoriju glede na morfometrijo in obseg njegovih razvojnih štadijev v življenskem ciklu za razvoj metod dobrega upravljanja s škodljivcem. Rezultati so pokazali, da samice ležejo jajčeca posamično na plodove, peclje, liste in cvetne brste. Po izleganju se ličinke zavrtajo v plodove, v katerih se hranijo s pulpo in jih s tem naredijo neprimerne za prodajo. Škodljivec ima pet razvojnih stopenj ličinke z različnim trajanjem, celotno obdobje ličinke je trajalo 32,20 ± 1,87 (SD) dni. Stopnji ličinke je sledilo obdobje bube, ki je trajalo 9,50 ± 1,84 (SD) dni. Življenska doba odraslih samcev in samic je bila 9,20 ± 1,73 (SD) in 13,20 ± 1,43 (SD) dni. Raziskava prispeva potrebne informacije za razvoj in uporabo različnih strategij nadzora pri integriranem upravljanju zavijača granatnega jabolka.

Ključne besede: granatno jabolko; *Deudorix epijarbas*; bionomika; morfometrija; razvojne stopnje ličinke

---

[1] University of Kashmir, Department of Zoology, Division of Entomology, Srinagar, Jammu and Kashmir, India
[2] Corresponding author, e-mail: inshayousuf444@gmail.com

1 INTRODUCTION

Pomegranate is an ancient sacred fruit belonging to genus *Punica* of family Punicaceae. It is regarded as a crop of arid and semi-arid regions because of its adaptability to diverse soil and climatic stresses. Pomegranate trees are valued for its products, which have immense nutritional, medicinal and industrial significance (Negi et al., 2003 and Sumner et al., 2005). However, this fruit tree is susceptible to damage inflicted by various insect pests reducing quality and quantity of its products (Ismail et al., 1989). Among them, the damage caused by pomegranate fruit borer, *Deudorix epijarbas* (Moore, 1858), is enormous throughout India. In Kashmir valley, this pest has been recorded as a serious and predominant pest of pomegranate fruits (Zaka- ur- Rab, 1980). During 20th century, pomegranate cultivation was carried out on large scale in the valley but its plantations succumbed to the attack by pomegranate butterfly. Nowadays, sporadic cultivation is prevalent in certain regions of Kashmir. The pest attack is from the onset of fruiting season til its completion. The larvae bore their way in pomegranate fruits and feed on pulp and seeds making the fruits sensitive to secondary infections of fungus and bacteria. In the fields, infested fruits are detected by the presence of holes on them, through which larvae exude their faecal matter, conferring these fruits an odious stink. As a result, the infested fruits become totally unfit for human consumption and thus impair their trade.

Recently, there is a renewed surge in pomegranate consumption following the assertion of its health benefits. It is deemed a rich source of antioxidants, vitamins, potassium, calcium, magnesium, iron and zinc as has been confirmed by findings of Gil et al.(2000) Aviram & Dornfeld (2001) and Haidari et al. (2009). Dietary supplementation with pomegranate is believed to prevent cancer (Afaq et al., 2005). Its fruits have antimicrobial, antimutagenic and antioxidant activities (Negi et al., 2003). Furthermore, it has been used in commercial cosmetic preparations on a large scale (Kim et al., 2002). As a result of this increased demand, new plantations are being established throughout the world. This results in higher risk of infestation of pomegranates by insect pests in general and by fruit borers in particular. Successful management strategy requires thorough knowledge on different aspects of insect pest. So far, little work is done on the bionomics of pomegranate fruit borer, *D. epijarbas* from Kashmir. Therefore, the present investigation was carried out to analyze the biology of *D. epijarbas* on pomegranate in laboratory at room temperature so as to replicate natural environmental conditions necessary for furnishing peak infestation of pomegranate fruit borer, for devising an opportune management strategy against the pest.

2 MATERIAL AND METHODS

To study the various aspect of biology, a stock culture of pomegranate fruit borer, *D. epijarbas* was maintained in Entomology Research Laboratory of Department of Zoology, University of Kashmir. The detailed methodology employed for the study is given below.

2.1 STOCK CULTURE OF *D. epijarbas*

The infested fruits were collected from Kashmir University Botanical Garden (KUBG) having geographic coordinates (34°07'40.66"N, 74°50'11.46"E and 5214ft) and Noor Bagh located at 34°04'27.26"N, 74°48'12.80"E and 5538 ft. The infested fruits were identified by the presence of entry holes made by larvae of this particular pest species. For studying different biological parameters of pomegranate fruit borer, *D. epijarbas* (Moore, 1858) in laboratory, the methodology of Bhumannavar and Viraktamath (2001) was followed. The collected fruits were then kept in large glass jars open at one end (Fig.1a) under ambient room temperature of 28.55 ± 2.83 °C and relative humidity of 75.15 ± 6.06 %. The open end of jar was covered with muslin cloth for ventilation and to prevent the attack of parasites and fungi. Pomegranate fruits were provided to these larvae and the exuded faecal matter was removed from these jars on daily basis (Fig. 1b). The larvae then transformed into pupae and were kept under same rearing conditions till the emergence of adults. The adult male and female butterflies were then shifted carefully to separate rearing jars, provided with fresh pomegranate twigs and calyx. Observations regarding duration of mating, preoviposition, egg laying (oviposition) and postoviposition were recorded under laboratory conditions. The fecundity and adult longevity of both male and female butterflies was also observed and recorded. Duration of different life stages *viz.* larval instars, pupation and adult stage was recorded from eggs laid in laboratory. The detailed data on morphometry of different stages of life cycle was also recorded. The cotton swabs soaked in 5 % sucrose solution served as a source of food and water for studying the behaviour of adult butterflies during the course of study.

2.1.2 STUDY OF DIFFERENT BIOLOGICAL PARAMETERS OF *D. epijarbas*

Observations with regard to different developmental stages were recorded from these freshly laid eggs on pomegranate twigs and calyx under laboratory conditions.

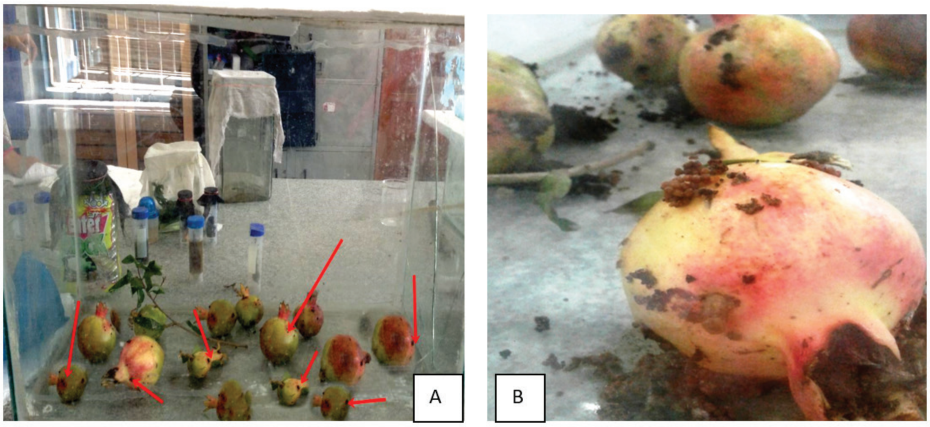


Figure 1: A: Rearing of fruit borer infested pomegranates in laboratory. B: Posterior region of *D. epijarbas* larva visible from the holes of infested fruits and exuded faecal matter.

2.1.2.1 Eggs

For the study of incubation period of eggs, 10 freshly laid eggs were taken from the stock culture and kept in separate Petri dish and observed daily till the emergence of young larvae. The observations on hatching percentage along with the changes in colour and shape of eggs were recorded as well.

2.1.2.2 Larval period

A total of 10 freshly emerged young larvae were transferred to separate glass jars using a fine camel hair brush and fresh food (pomegranate fruits cut into two halves) was provided to them on daily basis. These larvae were kept under constant observation to record the number and duration of different larval instar. The change in larval instar was substantiated by change in its colour which was further validated by presence of exuvium of head capsule on infested fruit. Duration of larval period (i.e. number of days taken by a larva since its emergence from egg up to its pupation) was also computed. In separate experiment, head capsule width for each larval instar was measured to separate different larval instars (Dyar, 1890). In the later stages, larvae stops feeding and become sluggish which signals the initiation of pupal stage.

2.1.2.3 Pupal period

Studies on duration of pupal period were carried out on 10 fully grown larvae that were kept in separate beakers and the number of days from termination of larval period till the appearance of adults were counted and recorded as pupal period. Observations regarding the colour and shape of pupae were also recorded.

2.1.2.4 Adult longevity

Total life span of adult i.e. number of days from emergence of adults to their death was regarded as longevity of adult butterflies. Observations on longevity were recorded for both male and female butterflies.

2.1.2.5 Ovipositional period and fecundity

To determine the ovipositional period, the eggs laid by female were observed and removed daily til the death of female butterfly. Fecundity is the total number of eggs laid by a single female butterfly during its lifetime. Both experiments were replicated 10 times.

2.1.2.6 Life cycle

Based on the duration of different stages in the life cycle of pomegranate fruit borer *viz.* egg, larva, pupae and adult time period required for the completion of life cycle was recorded..

2.1.2.7 Statistical analysis

The data obtained from the experiments was arranged in tables. The mean, range and Standard deviation of the readings was computed using SPSS software (16.0).

3 RESULTS

The observations on biology of *D. epijarbas* are presented under following headings.

3.1 EGGS

The female butterfly laid eggs singly on fruits, stalks, leaves and flower buds (Fig. 2a). These eggs were light greenish in colour when fresh, more or less rounded in shape and turned white just before hatching. The diameter of eggs ranged from 0.90 to 1.15 mm with an average of 1.04 mm ± 0.02 (SE) (Table 1). The incubation period of eggs varied from 8 to 12 days with a mean of 10.33 days ± 1.02 (SE) (Table 3).

3.2 LARVAL PERIOD

The destructive stage of this pest is represented by its different larval instars (Fig. 2b). The pest was found to pass through five larval instars on pomegranate fruits in laboratory before transforming into pupa. Head capsule width of each larval instar was used to determine the larval instar (Table 2). The duration of each larval instar and measurements of different developmental stages is given in Tables 3 & 4 respectively.

Newly hatched larvae were cylindrical, light brown in colour and possessing a triangular black structure on thoracic segments. The body of larva was covered with dense white hairs which were longer in abdominal segments. The developmental period of first larval instar ranged from 4 to 6 days with an average of 4.95 days ± 0.76 (SD) whereas the average body length and width was recorded as 3.94 mm ± 0.42 (SD) and 0.88 mm ± 0.08 (SD) respectively.

The second instar larva differed markedly from first instar in size and colour. This larval instar was dark brown in colour with less dense body hairs. Length of second larval instar of *D. epijarbas* ranged from 5.50 to 7.94 mm with an average of 6.79 ± 0.82 (SD) mm while the breadth ranged from 1.25 to 1.50 mm with a mean of 1.37 ± 0.08 (SD) mm (Table 4). Average duration of second instar larvae was found to be 5.45 ± 0.83 (SD) days, and ranged from 4.0-6.5 days.

Third larval instar was analogous to previous larval instar except in its dimensions. The developmental period of third instar larvae varied from 5 to 7.5 days with an average of 6.40 ± 0.87 (SD) days (Table 3). The length and width of this larval instar ranged from 9.30 to 12.95 mm with an average of 10.92 ± 1.24 (SD) mm,

Table 1: Morphometric measurements of egg of pomegranate fruit borer, *D. epijarbas*

Parameter	Observations										Mean (±SE)*	Range
Egg diameter (mm)	I	II	III	IV	V	VI	VII	VIII	IX	X	1.04 (±0.02)	0.90- 1.15
	1.00	1.02	1.15	0.90	1.09	1.04	1.07	1.03	1.06	1.05		

*Mean of 10 replications, figure in parenthesis are standard error.

Table 2: Head capsule width of different larval instars of pomegranate fruit borer, *D. epijarbas*

Larval instars	No. of larvae observed	Mean observed head width (± SE)	Range	Expected head width*	Difference
I	10	0.412 (± 0.017)	0.34-0.49		0
II	10	0.695 (± 0.025)	0.54-0.78	0.688	0.007
III	10	1.192 (± 0.015)	1.12-1.26	1.16	0.032
IV	10	1.770 (± 0.022)	1.66-1.86	1.99	0.218
V	10	2.447 (± 0.0345)	2.20-2.60	2.95	0.503

*Expected head capsule width established by Dyar's ratio (1.67mm). Multiplying Dyar's ratio with the observed head capsule width of 1st instar larva gives the expected head capsule width of 2nd larval instar which when multiplied again with Dyar's ratio gives expected head capsule width of 3rd larval instar and so on.

Mean observed head capsule width of 1st larval instar (N = 10) = 0.412mm
Mean observed head capsule width of 2nd larval instar (N = 10) = 0.695mm
Growth ratio (Dyar's ratio) = Head capsule width of 2nd larval instar/ Head capsule width of 1st larval instar = 0.695/ 0.412 = 1.67mm

and 2.15 to 2.89 mm with an average of 2.50 ± 0.26 (SD) mm respectively.

The colour of body became considerable darker in stout fourth instar larvae. Average duration of fourth larval instar was 7.10 ± 0.39 (SD) days whereas mean body length was 15.58 ± 0.80 (SD) mm with an average width of 3.63 ± 0.31 (SD) mm.

Fully mature larva was stouter, slothful and dark green in colour and the final instar lasted for 8.35 ± 0.58 days (7.5 to 9.0 days). Average body length of fifth larval instar was 19.54 ± 1.53 (SD) mm (17.00 to 21.72 mm) whereas the average breadth varied from 5.38 to 6.77 mm with an average of 5.90 ± 0.50 (SD) mm. Thus, the total larval period of *D. epijarbas* on pomegranates in laboratory ranged between 29.0 to 35.0 days with an average of 32.20 (± 1.87) days (Table 3).

A fully matured larva of pomegranate fruit borer impeded its feeding, became smaller in size and got transformed into pre- pupal stage. The pre pupa was dark green in colour with brown head. The duration of this stage varied from 2.5 to 3.5 days with an average of 3.00 ± 0.41 (SD) days.

3.3 PUPAL PERIOD

The pupa of *D. epijarbas* was initially light brown in colour and gradually changed its colour to dark brown (Fig. 2c). The length of pupal stage varied from 13.22 to 16.00 mm and average length was found to be 14.57 ± 0.92 (SD) mm. The pupal breadth ranged between 6.00 and 6.96 mm with an average of 6.55 ± 0.29 (SD) mm. The duration of pupal stage varied from 7.0 to 12.0 days with a mean of 9.50 ± 1.84 (SD) days. Pupation was observed to occur on or inside the damaged fruits as well as on the sides and corners of

Table 3: Duration of different developmental stages of *D. epijarbas* in laboratory

Parameters	No. of observations	Range	Mean±SD
Incubation period	10	7.0-11.0	8.75 (± 1.38)
1st larval instar	10	4.0-6.0	4.95 (± 0.76)
2nd larval instar	10	4.0-6.5	5.45 (± 0.83)
3rd larval instar	10	5.0-7.5	6.40 (± 0.87)
4th larval instar	10	6.5-7.5	7.10 (± 0.39)
5th larval instar	10	7.5-9.0	8.35 (± 0.58)
Total larval period	10	29.0-35.0	32.20 (± 1.87)
Pre- pupa	10	2.5-3.5	3.00 (± 0.41)
Pupa	10	7.0-12.0	9.50 (± 1.84)
Male adult longevity	10	7.0-11.5	9.20 (± 1.73)
Female adult longevity	10	11.0-15.0	13.20 (± 1.43)
Total life span (Male)	10	58.5-69.5	62.65 (± 3.18)
Total life span (Female)	10	62.0-74.5	66.65 (± 3.68)

SD = Standard deviation, figures in parenthesis are standard deviation of mean

Table 4: Measurements of different life stages in pomegranate fruit borer, *D. epijarbas* under laboratory conditions

Developmental stage	Mean length (± SD)*	Range	Mean breadth (± SD)*	Range
1st larval instar	3.94 (± 0.42)	3.50-4.50	0.88 (± 0.08)	0.75-1.00
2nd larval instar	6.79 (± 0.82)	5.50-7.94	1.37 (± 0.08)	1.25-1.50
3rd larval instar	10.92 (± 1.24)	9.30-12.95	2.50 (± 0.26)	2.15-2.89
4th larval instar	15.58 (± 0.80)	14.44-16.70	3.63 (±0.31)	3.07-4.20
5th larval instar	19.54 (± 1.53)	17.00-21.72	5.90 (± 0.50)	5.38-6.77
Pupa	14.57 (± 0.92)	13.22-16.00	6.55 (± 0.29)	6.00-6.96

*Mean of 10 replication/ developmental stage, figures in parenthesis are standard deviation.

Table 5: Measurements of adult male and female *D. epijarbas*

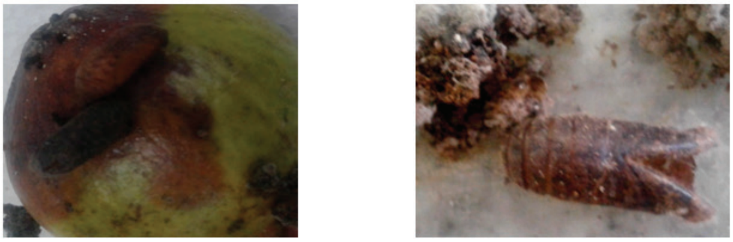
Adult	Mean body length in mm (± SD)*	Range	Mean wing expanse in mm (± SD)*	Range
Male	18.54 (± 1.57)	16.50-20.93	38.25 (± 1.52)	36.40-40.50
Female	22.97 (±1.07)	21.65-24.66	42.26 (± 1.26)	40.58-44.50

*Mean of 10 replication/ individual, figures in parenthesis are standard deviations.

Table 6: Duration of different biological phenomenon in the life cycle of pomegranate fruit borer, *D. epijarbas*

Observations (N)	Mating (minutes)	Pre-oviposition (days)	Oviposition (days)	Post-oviposition (days)	Fecundity(number)
I	59	3	3.5	7.5	27
II	57	3.5	4	4	24
III	65	4	3	5.5	23
IV	66	3	4.5	4	29
V	58	4	4.5	5	20
VI	63	4	3	6.5	25
VII	62	3.5	3	6	29
VIII	64	3	3.5	5	22
IX	59	3	4	7	26
X	57	3.5	3.5	5.5	28
Mean ± SD	61.00 ± 3.40	3.65 ± 0.44	3.65 ± 0.58	5.6 ± 1.17	25.30 ± 3.06

a. Egg b. Larva



c. Pupa d. Empty pupal case

e. Adult

Figure 2 (a - e): Different developmental stages in the life cycle of *D. epijarbas*.

rearing jar. Adult butterflies emerged by splitting the pupal covering dorsally (Fig. 2d and 2e).

3.4 ADULTS

Sexual dimorphism is conspicuous in adult pomegranate fruit borer, *D. epijarbas*. The female butterflies were larger in size with longer wing expanse than their male counterparts. The females were dull brown in colour and measured 22.97 ± 1.07 (SD) mm (Table 5). Average width of female butterfly including wing span was observed to be 42.26 ± 1.26 (SD) mm with longevity of 13.20 ± 1.43 (SD) days. The males were brick red in colour having an average body length of 18.54 ± 1.57 (SD) mm. The width across wings was found to be 38.25 ± 1.52 (SD) mm and longevity of 9.20 ± 1.73 (SD) days (Table 5).

The preoviposition, oviposition and post oviposition period of female *D. epijarbas* ranged from 3 to 4 days with an average of 3.45 ± 0.44 (SD) days, 3 to 4.5 days with an average of 3.65 ± 0.58 (SD) days and 4 to 7.5 days with an average of 5.6 ± 1.17 (SD) days respectively. Fecundity of pomegranate fruit borer ranged between 20 to 29 eggs with a mean of 25.30 ± 3.06 (SD) eggs (Table 6).

The total life cycle of *D. epijarbas* in laboratory was observed to vary from 58.5 to 69.5 days for males and mean of 62.65 ± 3.18 (SD) days while on the contrary, the total life cycle of females ranged from 62.0 to 74.5 days with a mean of 66.65 ± 3.68 (SD) days (Table 3).

4 DISCUSSION

A number of studies have been carried on the biological parameters of *Deudorix (=Virachola) isocrates* (Fabricius, 1793) by various workers *viz.* Kabre and Mohalkar (1992), Thirumurugan (1992), Karuppuchamy et al. (1998), Singh & Singh (2001), Tiwari & Mishra (2007), Bhut et al. (2013) and Chettry et al. (2015). However, there are reports of preliminary studies carried out on biology of *D. epijarbas*, an allied species of *D. isocrates,* by Zak- ur- rab (1980), Verma (1985) and Dutta (1992).

In the present experiments, mean incubation period of 8.75 ± 1.38 days was recorded for the eggs of *D. epijarbas*. Gharbi (2010) reported a lesser incubation period of 5.23 ± 0.32 days for another species of pomegranate fruit borer, *V. livia* Klug, 1834 in Tunisia. This disparity may be because of difference in pest species, variability of host plant, agroclimatic conditions or latitudinal clines of place at which the study was carried out.

The present study on the biology of pomegranate fruit borer, *D. epijarbas* has shown that larvae of the pest pass through five larval instars on pomegranates in laboratory. Change in larval instars was accomplished on the basis of head capsule width of larvae, which unlike average larval weight did not overlap and thus was an accurate indicator of larval development. In present investigation, total larval period of *D. epijarbas* varied between 29.0 and 35.0 days with an average of 32.20 ± 1.87 (SD) days. These results are somewhat in agreement with the findings of Dutta (1992) and Mohi- ud- din et al. (2014) who both worked on same species of pomegranate fruit borer in Kashmir. At the end of larval period, larvae entered a non- motile, non- feeding pre- pupal phase that lasted for 3 days preceding the formation of pupa.

Our study revealed that pupation occurred inside or on the skin of fruit, at corners and sides of rearing jar and lasted for 7 to 12 days with a mean of 9.50 ± 1.84 (SD) days. Similar observations were made by Tiwari & Mishra (2007) and Chettry et al. (2015) for *D. isocrates* on different hosts implying that plant fed to larvae during rearing, has no effect for pupal development of genus *Deudorix*.

The present study showed that there was a difference in female and male longevity. In our experiments, adult females lived longer than adult males with mean longevity of 13.20 and 9.20 days respectively. The average life span of males was found to be 62.65 ± 3.18 (SD) days (58.5 to 69.5 days), whereas females had average life span of 66.65 ± 3.68 days (62.0 to 74.5 days). Variations in colour patterns of male and female butterfly found in the present study are in consistency with the previous studies of Zaka- ur- rab (1980), Verma (1985) and Dutta (1992).

Our study revealed that body length in males ranged from 16.50 to 20.93 mm with an average of 18.54 ± 1.57 (SD) mm whereas for females, average body length was 22.97 ± 1.07 (SD) mm (21.65 to 24.66 mm). The average width across wings in male was 38.25 ± 1.52 (SD) mm (36.40 to 40.50 mm) and in female, wing expanse ranged from 40.58 to 44.50 mm with a mean of 42.26 ± 1.26 (SD) mm. These results are in line with the observations of Singh & Singh (2001) and Tiwari & Mishra (2007) about body length and wing expanse for a different species of pomegranate fruit borer, *D. isocrates* on aonla, *Phyllanthus emblica* L..

The present study revealed that the mean pre-oviposition, oviposition and postoviposition period of *D. epijarbas* in laboratory were 3.45 ± 0.44 (SD), 3.65 ± 0.58 (SD) and 5.60 ± 1.17 (SD) days respectively. From our study, the egg laying capacity of female butterfly was found to vary from 20 to 29 eggs with a mean of 25.30 ± 3.06 (SD) eggs. Conversely, the study of Zaka- ur- rab (1980) have shown that female butterfly laid only 4 eggs. Total life cycle was worked out to take 62.65 days in males and 66.65 days in females, implying that fruit borer can complete 2- 4 generations during fruiting season of pomegranate.

5 CONCLUSIONS

The present study thus provides important information about development, longevity and other biological parameters of *D. epijarbas*. In the present study, mean diameter of eggs of the pest was found as 1.04 (± 0.02) mm. Further, the duration of 1st, 2nd, 3rd, 4thand 5th larval instars was 4.95 (± 0.76), 5.45 (± 0.83), 6.40 (± 0.87), 7.10 (± 0.39) and 8.35 (± 0.58) days respectively. The female butterflies showed longer adult longevity (13.20 ± 1.43 days) as well as total life span (66.65 ± 3.68 days) than their male counterparts with adult longevity of 9.20 (± 1.73) days and total life span of 62.65 (± 3.18) days. The body length and wing expanse was recorded as 18.54 (± 1.57) mm & 38.25(± 1.52) mm in males and 22.97 (± 1.07) mm and 42.26 (± 1.26) mm in females. The duration of preoviposition, oviposition and postoviposition was 3.65 (± 0.44), 3.65 (± 0.58) and 5.60 (± 1.17) days respectively. The present understanding of the biology of this particular pest species would help in its management and control. During the current experiments, a gap of few days was observed between oviposition and penetration of fruits by first instar larva which may prove significant while formulating its control strategies. Therefore, further studies should try to exploit this gap and during the assessment of different control strategies focus should be on preventing the entry of larvae in pomegranate fruits.

6 REFERENCES

Afaq, F., Saleem, M. and Mukhtar, H. (2003). Pomegranate fruit extract is a novel agent for chemoprevention: Studies in mouse skin. *2nd Annual AACR International Conference on Frontiers in Cancer Prevention Research, 26- 30 October. Cancer Epidemiology and Biomarkers and Prevention, 12,* 1351-1353. https://doi.org/10.1016/S0021-9150(01)00412-9

Aviram, M. and Dornfeld, L. (2001). Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. *Atherosclerosis, 158*, 195- 198.

Bhumannavar, B. S. and Viraktamath, C. A. (2001). Rearing techniques for three species of *Othreis* (Lepidoptera: Noctuidae) and their ectoparasitoid, *Eupletrus maternus* Bhatnagar (Hymenoptera: Eulophidae). *Journal of Biological Control, 15*(2), 189- 192.

Bhut, J. B., Borad, P. K. and Gadhiya, H. A. (2013). Bionomics and evaluation of different biocides against anar butterfly, *Virachola isocrates* (Fabricius) infesting pomegranate. *International Journalof Plant Protection, 6*(2), 338-343.

Chhetry, M., Gupta, R. and Tara, J.S. (2015). Bionomics of *Deudorix isocrates* Fabricius (Lepidoptera: Lycaenidae), a new potential host of sweet orange, *Citrus sinensis* L. Osbeck in J & K, India. *International Journal Science and Nature, 6*(2), 238-241.

Dutta, T. S. (1992). *Biology and management of pomegranate fruit borer, Deudorix epijarbas (Moore)*. Thesis submitted to Sher- e- Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Srinagar.

Dyar, H. G. (1890). The number of moults of Lepidopterous larvae. *Psyche, 5*, 420-422. https://doi.org/10.1155/1890/23871

Gharbi, N. (2010). Laboratory rearing of pomegranate fruit butterfly *Virachola livia* on two host plants in Tunisia. *Tunisian Journal Plant Protection, 5*, 195-199.

Gil, M. I., Tomas- Barberan, F. A., Hess- Pierce, B., Holcroft, D. M. and Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural & Food Chemistry, 48*, 4581-4589. https://doi.org/10.1021/jf000404a

Haidari, M., Ali, M., Casscells, S.W. and Madjid, M. (2009). Pomegranate *(Punica granatum)* purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir. *Phytomedicine, 16*(12), 1127-36. https://doi.org/10.1016/j.phymed.2009.06.002

Ismail, I. I., El- Nagar, S. and Attia, A. A. (1989). The aphid fauna of fruit trees in Egypt. *African Journal Agricultural Science, 13*(1-2), 1-7.

Kabre, G. B. and Moholkar, P. R. (1992). Studies on life history of pomegranate fruit- borers, *Virachola isocrates* Fabr. *Journal of Insect Science, 5*(2), 213.

Karuppuchamy, P., Balasubramaniam, G. and Babu, P. C. S. (1998). The biology of pomegranate fruit borer, *Deudorix isocrates*. *The Madras Agricultural Journal, 5*(6), 252-256.

Kim, N. D., Mehta, R., Yu, W., Neeman, I., Livney, T., Amichay, A., Poirier, D., Nicholls, P., Kirby, A., Jiang, W., Mansel, R., Ramachandran, C., Rabi, T., Kaplan, B. and Lansky, E. (2002). Chemopreventive and adjuvant therapeutic potential of pomegranate *(Punica granatum)* for human breast cancer. *Breast Cancer Research and Treatment, 71*, 203-217. https://doi.org/10.1023/A:1014405730585

Mohi- ud- din, S. (2014). *Current status, life cycle and development of IPM module of fruit borer, Deudorix epijarbas (Moore) on pomegranate*. Thesis submitted to Sher- e- Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Srinagar.

Murugan, M. and Thirumurugan, A. (2001). Studies on eco-friendly approaches to manage pomegranate fruit borer. *Plant Protection. Bulletin, 5*(3), 13-15.

Negi, P. S., Jayaprakasha, G. K. and Jena, B. S. (2003). Antioxidant and antimutagenic activities of pomegranate peel extracts. *Food Chemistry, 80*, 393-397. https://doi.org/10.1016/S0308-8146(02)00279-0

Singh, H. B. and Singh, S. B. (2001). Biology of *Deudorix isocrates* on its new potential host, aonla. *Indian Journal of Entomology, 63*(1), 19-25.

Sumner, M. D., Elliott- Eller, M., Weidner, G., Daubenmier, J. J., Chew, M. H., Marlin, R., Raisin, C. J. and Ornish, D. (2005). Effects of pomegranate juice consumption on myocardial perfusion in patients with coronary heart disease. *American*

Journal Cardiology, 96, 810-814. https://doi.org/10.1016/j.amjcard.2005.05.026

Thirumurugan, A. (1992). *Studies on insect pests of pomegranate and their control.* M. Sc. (Ag.) Thesis, Agricultural College and Research Institute (T. N. A. U.), Killikulam T. N. (India).

Tiwari, A. K. and Mishra. P. (2007). Biology of *Deudorix isocrates* Fabricius on aonla, *Emblica officinalis. Annals of Plant Protection Science, 15*(2), 335-337.

Verma, R. R. (1985). Preliminary observations on the biology of pomegranate butterfly, *Deudorix epijarbas* Moore. *Agricultural Science Digest, 5*, 1-2.

Zaka- ur- Rab, M. (1980). The cornelian *Deudorix epijarbas* Moore (Lepidoptera: Lyaenidae) as a serious pest of pomegranate fruits in Kashmir. *Journal of Entomological Research, 4*, 233-235.

doi:10.14720/aas.2020.116.1.1232 Original research article / izvirni znanstveni članek

Comparative effects of cantharidin and endothall on gene expression and activity of antioxidant enzymes in *Cichorium intybus* L.

Yalda GHOTBZADEH KERMANI [1] , Ramazan Ali KHAVARI NEJAD [1], Homa MAHMOODZADEH [2, 3],
Khadijeh SHAHROKH ABADI [2]

Received July 24, 2019; accepted September 04, 2020.
Delo je prispelo 24. julija 2019, sprejeto 04. septembra 2020.

Comparative effects of cantharidin and endothall on gene expression and activity of antioxidant enzymes in *Cichorium intybus* L.

Abstract: Cantharidin and its analog, endothall, are known to have phytotoxic effects and their mechanism of action involves the inhibition of phosphatases. Enzymes and antioxidant compounds act as barriers against phytotoxic compounds. Catalase and peroxidase are among the most important antioxidant enzymes. *Cichorium intybus* L. has traditionally been used for its medicinal properties and contains various phytochemical and enzymatic compounds. The present study aimed to investigate the comparative effects of cantharidin and endothall with concentration of 2.5, 5.5 and 10 µg ml^{-1} on the changes in the gene expression of catalase and glutathione peroxidase. Furthermore, we assess the activities of these enzymes in the shoots and roots of *Cichorium intybus* L.. According to the findings, the expression of catalase and glutathione peroxidase increased in the samples treated with cantharidin more than endothall compared to the controls in both shoot (the most significant is in cantharidin with 2.5 µg ml^{-1} concentration) and root samples (the most significant is in cantharidin with 5.5 µg ml^{-1} concentration). In addition, the activity of catalase and concentrations of cantharidin (2.5 µg ml^{-1}) in shoot samples led to the more intense stimulation of catalase and glutathione peroxidase compared to root samples. We suggest that cantharidin and endothall have negative effect on expression and absorption of antioxidant enzymes.

Key words: cantharidin; catalase; *Cichorium intybus* L.; endothall; glutathione reroxidase

Primerjalni učinki kantaridina in endotala na izražanje genov in aktivnost antioksidacijskih encimov pri navadnem potrošniku (*Cichorium intybus* L.)

Izvleček: Kantaridin in njegov analog endotal sta poznana po fitotoksičnih učinkih, pri katerih njun mehanizem delovanja vsebuje inhibicijo fosfataz. Encimi in antioksidacijske spojine tvorijo bariero proti fitotoksičnim spojinam. Katalaza in peroksidaza sta med najpomembnejšimi antioksidacijskimi encimi. Navadni potrošnik (*Cichorium intybus* L.) se tradicionalno uporablja kot zdravilna rastlina zaradi vsebnosti različnih fitokemikalij in encimov. V raziskavi so bili preučevani primerjalni učinki kantaridina in endotala v koncentracijah 2,5, 5,5 in 10 µg ml^{-1} na spremembe v izražanju genov za katalazo in glutation peroksidazo. Aktivnosti teh dveh encimov so bile ocenjene v poganjkih in koreninah navadnega potrošnika. Izsledki so pokazali, da se je izražanje genov za katalazo in glutation peroksidazo bolj povečalo v vzorcih, ki so bili tretirani s kantaridinom kot v tistih tretiranih z endotalom v primerjavi s kontrolo, tako v poganjkih (najznačilnejši učinek je imela koncentracija kantaridina 2,5 µg ml^{-1}) kot v koreninah (najznačilnejši učinek je imela koncentracija kantaridina 5,5 µg ml^{-1}). Dodatno sta aktivnost katalaze in koncentracija kantaridina (2,5 µg ml^{-1}) v vzorcih poganjkov vodili k intenzivnejši stimulaciji katalaze in glutation peroksidaze v primerjavi z vzorci korenin. Predpostavljamo, da imata kantaridin in endotal negativni učinek na izražanje genov antioksidacijskih encimov in na njihovo absoprcijo.

Ključne besede: kantaridin; katalaza; *Cichorium intybus* L.; endotal; glutatione peroksidaza

---

[1] Islamic Azad University, College of Basic Science and Research Branch, Department of Botany, Tehran, Iran
[2] Islamic Azad University, Mashhad branch, Department of Biology, Mashhad, Iran
[3] Corresponding author, e-mail: homa_mahmoodzadeh@yahoo.com

1 INTRODUCTION

Cichorium intybus L. belongs to the Asteraceae family (Heimler et al., 2009) and Wild chicory or blue daisy *C.intybus* is a diploid plant species (2n = 18) (Funk et al., 2005). The shoots of *C.intybus* could be as high as 0.5-1.5 meters in the wild state, and they may even exceed two meters. It is cultivated as an ornamental and medicinal plant across Europe. In Iran, *C. intybus* grows in mountainous regions, as well as Khorasan, Guilan, Mazandaran and Tehran provinces.

Chicory is an important medicinal plant which accumulates various specific organic compounds, such as storage polysaccharide inulin, sesquiterpene lactones, cumarins, phenolic acids and flavonoids (Bais & Ravishankar, 2001; Senderski, 2009). Flavonoids and terpenoids are among the important compounds found in *C. intybus* . Some cultivars of this plant are widely cultivated in countries such as the United States, India, and New Zealand as a fodder plant to feed livestock. Furthermore, *C. intybus* has been traditionally used as a medicinal herb. As such, the phytochemical and enzymatic compounds found in *C. intybus* are of almost importance (Abbas et al., 2015). Cantharidin is a toxic terpenoid compound, which is found in the beetles of Meloidae and Oedemeridae families. This natural terpenoid is also produced by the blister beetle (*Epicauta* spp.) and Spanish flies (*Lytta vesicatoria* (L., 1758)) as a protection mechanism (Bajsa et al., 2011). Cantharidin has been used as a medicine in China since 2000 years ago. This compound is of medical and economic significance due to its medicinal proprieties and severe toxicity in livestock. Nevertheless, its usage has recently become restricted in medicine due to its high toxicity and adverse effects (Young, 2017). Previous studies have denoted that cantharidin affects cells through inhibiting phosphatases proteins (PPTs) 1 and 2. Phosphatases are involved in various cellular functions through phosphorylation-dephosphorylation and are responsible for conducting phosphorylation-dependent signals. The other mechanisms in this regard include activating apoptosis and blocking all the apoptosis inhibitors (Ward et al., 2017). In addition, PPTs control several plant cell functions, such as pathway crosstalk, light perception, response to pathogens, and hormonal functions (Ward et al., 2017a; Rikin & Rubin, 1993). Considering the effectiveness of PPTs, they have been applied to obtain various compounds for special purposes. Endothall herbicide is one of the analogs of these compounds (Duke & Powles, 2008).

Endothall (7-oxabicyclo heptane-2,3-dicarboxylic acid) was introduced in the 1950 with the commercial name ACCELERATE (Duke & Powles, 2008a). Despite its history, endothall remains one of the several herbicides to be used widely. Considering its mechanism of action, endothall inhibits the entrance of leucine amino acid into the protein structure in different parts of plants. At high concentrations, endothall could even inhibit the synthesis of lipids. The plants affected by this toxin die in a browned form and decrease the light genes expression. According to the WSSA Herbicide Handbook, endothall affects the cell membrane, as well as a wide range of molecular pathways, which are not clearly identified (Dayan & Watson, 2011). In addition, previous findings have indicated that cantharidin and endothall are both specific inhibitors of phosphatases proteins, and the phosphatase protein functioning in plants is inhibited more effectively by endothall compared to cantharidin (Bajsa et al., 2012).

The inhibition of phosphatases proteins in plants could damage the plant structure. As such, plants have developed various strategies to respond to the herbicide toxicity. For instance, the antioxidant system is a considerable defense mechanism in plants, which is activated through focused responses with the involvement of various enzymes, including superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, glutathione reductase, and glutathione transferase. Catalase is an important enzyme, which is responsible for a major part of the antioxidant defense in plants. Moreover, reports have suggested that following catalase, peroxidase plays a key role in the removal of hydrogen peroxide (Bajsa et al., 2012a).

To date, no studies have evaluated the effects of cantharidin and endothall on the gene expression of antioxidant enzymes, as well as the changes in the activities of catalase and glutathione peroxidase proteins. Considering the adverse effects of these herbicides on cell physiology (fodder and medicinal plants), the present study aimed to investigate the effects of cantharidin and endothall on the expression of catalase and glutathione peroxidase antioxidant genes in *Cichorium intybus* (L.).

2 MATERIALS AND METHODS

2.1 CULTIVATION, TREATMENT, AND SAMPLING OF CICHORIUM INTYBUS (L.)

In this study, the seeds of *Cichorium intybus* L. were obtained from Pakan Bazr Company in Isfahan, Iran. The seeds were placed in suitable pots for germination, and daily irrigation was performed for five days with 200 ml of water per week. After this period and when the plants were in the two-leaf stage, treatment of the samples was initiated using 200 ml week^{-1} of each concentration, and plants were water-sprayed every other day.

In order to determine the proper concentrations of cantharidin and endothall, the plants were randomly treated with 10, 50, 100, and 150 µg ml^{-1}, and the results of the initial cultivation indicated that the plants had no germination at the other concentrations than 10 µg ml^{-1}. Therefore, the concentration range of less than 10 µg ml^{-1} was selected for the main experiments. After determining the proper concentrations, cultivated seeds were treated by 2.5, 5.5 and 10 µg ml^{1-} for endothall and cantharidin respectively.

Plant treatments were performed using 400 ml of each treatment. Experiments were conducted in controlled laboratory conditions (25 ± 1 C°, 16:8 h (L : D), and harvesting was accomplished after two months of vegetative growth. At the next stage, separate samples were obtained from the roots and shoots of plants.

2.2 RNA EXTRACTION AND CDNA SYNTHESIS

Changes in the expression of catalase and glutathione peroxidase genes were examined in the shoots and roots (2 cm) of the samples. RNA extraction was performed using a Fermentas kit in accordance with the instructions of the manufacturer. Nanodrop was used to assess the presence and evaluate the purity of the extracted RNA. To remove the DNA impurities, we applied the DNase enzyme (Fermentas DNase I, RNase-free) in accordance with the protocol.

At the next stage, the cDNA was produced from the extracted RNA using RevertAid First Strand cDNA kit based on the proposed method in its protocol (According to the Fermentas kit). Eventually, the cDNA was used as the model DNA in polymerase chain reaction (PCR).

2.3 EXAMINATION OF THE CHANGES IN THE EXPRESSION OF THE CATALASE AND GLUTATHIONE PEROXIDES GENES USING QRT-PCR

Real-time PCR was employed to examine the changes in the expression of catalase and glutathione peroxidase genes using a PCR device (Light Cycler, Bio Rad) and SYBR Green color with the reaction volume of 20 ml, which consisted of 10 ml of the master mix containing SYBR Green, two ml of the left primers, and two ml of the right primers (Table 1).

The duration of the cycles was set at three minutes at the temperature of 95 °C before 40 cycles (10 seconds at 95 °C), and the final stage lasted for 30 seconds at the temperature of 60 °C in accordance with the protocol of the SYBR Green kit (Sanders & Lee, 2013). Eventually, the melting curve was drawn, and the changes in the expression of catalase and glutathione peroxidase genes were compared with the control samples.

2.4 MEASUREMENT OF ENZYMATIC ACTIVITY

To measure the activity of enzymes, 0.5 g of the fresh shoot or root tissue of the plant was milled in liquid nitrogen. Afterwards, 50 mM of phosphate buffer containing 1 % polyvinyl pyrrolidine and EDTA (1mM) were added to the milled tissues, and the mixture was centrifuged at the temperature of 4 °C for 20 minutes at 14,000 g. The supernatant fluid was collected to measure the proteins and preserved at the temperature of -20 °C.

2.5 MEASURING THE ACTIVITY OF CATALASE

To measure the activity of catalase, a reaction mixture composed of 20 ml of the extract, 980 ml of phosphate buffer (50 mM), and oxygenated water (15 mM) was prepared, and the reaction was rapidly initiated by adding oxygenated water. Reduced absorption of hydrogen peroxide was recorded as the changes in absorption over time at the wavelength of 240 nanometers. The enzyme activity was expressed in terms of µmol g^{-1} of fresh tissue per minute (Marschner & Cakmak, 1992).

2.6 MEASURING THE ACTIVITY OF GLUTATHIONE PEROXIDASE

The activity of glutathione peroxidase was measured based on the reduction of oxidized glutathione by the glutathione reductase enzyme with the consumption of nicotinamide adenine dinucleotide phosphate (NADPH). To perform the experiment, the reaction mixture containing 250 ml of potassium phosphate buffer (100 mM), 120 ml of NADPH (1 mM), 50 ml of oxidized glutathione (10 mM), and 480 ml of distilled water

Table 1: Sequence of utilized primers(primers are designed according to the genes used, from the NCBi database)

Gene	Right Sequence Primer	Left Sequence Primer
Catalase	CATCTGATTGAAAAAGCTTGCG	TTTGAGGGCTTCCAGGTTG
Glutathione Peroxidase	GTCACAAGAACACCGCTTT	CGCAACAATTCGCCTT

was prepared. Following that, 100 ml of the root or shoot extract of the plant were added to the reaction mixture and assorted.

At the next stage, the changes in the absorption were read at 340 nanometers for 120 s based on the kinetic method using a spectrophotometer. The enzyme activity was expressed based on the changes in the absorption min^{-1} mg^{-1} protein (Mac Adam, 1992).

2.7 STATISTICAL ANALYSIS

The experiments were run in a fully random design format with three replications. Data analysis was performed by SPSS 22.0 software, and the minimum significance level of $p \leq 0.05$ was considered to compare the means.

3 RESULTS AND DISCUSSION

3.1 EVALUATING THE CHANGES IN THE EXPRESSION OF THE CATALASE AND GLUTATHIONE PEROXIDASE GENES USING QRT-PCR

3.1.1 Changes in the expression of the catalase and glutathione peroxidase genes in the shoots of the samples treated with cantharidin and endothall

According to the investigation of the changes in the expression of catalase in the samples treated with endothall, the changes in the expression of this gene were significant at the concentration of 5.5 µg ml^{-1} compared to the controls. Furthermore, the changes in the catalase gene in the samples treated with cantharidin were considered significant at the concentrations of 2.5 µg ml^{-1}. In the samples treated with cantharidin, the catalase gene had a more significant elevated expression compared with the samples treated with endothall.

According to the investigation of the changes in the glutathione peroxidase gene in the samples treated with endothall, the expression of this gene increased in the shoots of the samples treated with 2.5 µg ml^{-1} of endothall compared to the control. Moreover, in the shoot samples of the plants treated with cantharidin, the elevation of the changes in the expression of this gene was observed at the concentrations of 2.5 and 10 µg ml^{-1}. Comparison of the changes in the expression of the peroxidase gene in the samples treated with cantharidin and endothall indicated that the increase in the expression of this antioxidant gene was more significant with cantharidin.

3.1.2 Changes in the expression of the catalase and glutathione peroxidase genes in the roots of the samples treated with cantharidin and endothall

According to the investigation of the changes in the expression of the catalase gene in the roots of the samples treated with endothall, the changes in the expression of this gene were significant at the concentration of 10 µg ml^{-1} compared to the controls, while no significant changes were denoted at the other concentrations compared to the control. On the other hand, the results of the changes in the glutathione peroxidase and catalase genes in the samples treated with cantharidin indicated that the expression of these genes had a significant difference compared to the controls. Moreover, the expres-

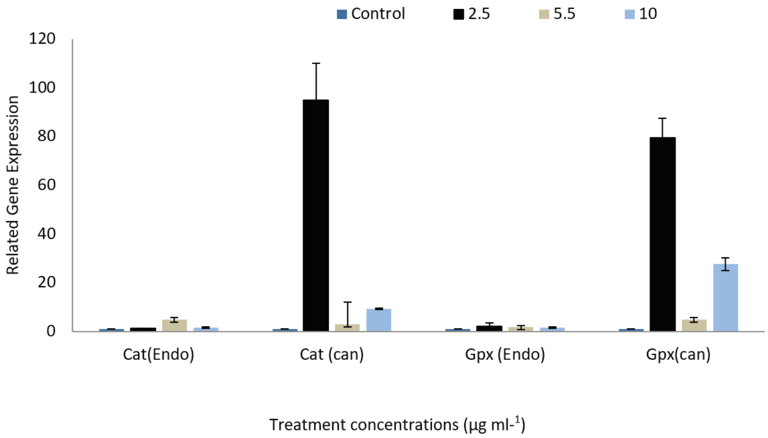


Figure 1: Changes in the expression of catalase and glutathione peroxidase antioxidant genes in the shoot of *Chicorium intybus* L. treated with endothall and cantharidin (µg ml^{-1})

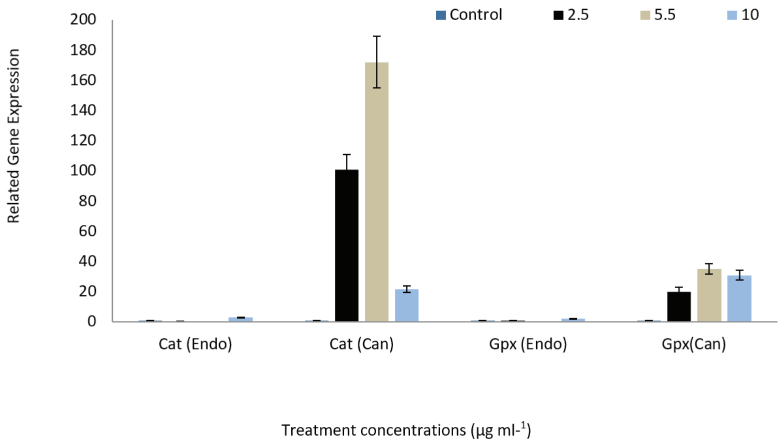


Figure 2: Changes in the expression of catalase and glutathione peroxidase antioxidant genes in the root of *Chicorium intybus* L. treated with endothall and cantharidin (µg ml^{-1})

sion of these genes increased in the roots of the samples treated with 5.5 µg ml^{-1} of cantharidin compared to the controls. Comparison of the changes in the expression of the peroxidase and catalase genes in the samples treated with cantharidin and endothall demonstrated that the increased expression of these antioxidant genes was more significant with cantharidin.

3.2 THE ACTIVITY OF CATALASE AND GLUTATHIONE PEROXIDASE ENZYMES

3.2.1 Measurement of the absorption of catalase and glutathione peroxidase in the shoot of the samples treated with cantharidin and endothall

Comparison of the mean absorption of catalase in the shoots of the samples treated with endothall indicated that the mean absorption of catalase in the shoots had a significant difference with the controls at the concentrations of 5.5 µg ml^{-1}, while the mean absorption showed no significant difference at the concentrations of 2.5 and 5.5 µg ml^{-1}.

Comparison of catalase absorption in the shoots of the samples treated with cantharidin showed a significant difference in the mean absorption of this enzyme between the shoots of the control samples and those treated with the concentrations of 2.5 µg ml^{-1}, then 10 µg ml^{-1}. As can be observed, changes in the cantharidin concentration increased the mean absorption of catalase in the shoots of the plants compared to the controls. According to the comparison of the activity of catalase in the samples treated with cantharidin and endothall, the maximum changes in the activity of this enzyme were observed in the samples treated with cantharidin.

The mean absorption of glutathione peoxidase was considered significant in the shoots of the *Cichorium intybus* L. samples treated with endothall at all the concentrations compared to the controls. The maximum changes in the samples treated with endothall were observed in the samples treated with 5.5 µg ml^{-1}. Furthermore, the activity of glutathione peroxidase increased significantly in the shoots of the samples treated with cantharidin compared to the controls. In addition, the maximum enzyme activity was observed at the concentration of 2.5 µg ml^{-1} in these treatment groups. Comparison of the activity of glutathione peroxidase in the samples treated with cantharidin and endothall indicated that the maximum changes in the activity of this enzyme were observed in the samples treated with cantharidin.

3.2.2 Measurement of the absorption of catalase and glutathione peroxidase in the roots of the samples treated with cantharidin and endothall

Comparing the mean absorption of catalase in the root treated with endothall indicated that the mean absorption of catalase in the root indicated a significant difference with the control at the concentration of 10 µg ml^{-1} other concentrations did not show any significant difference. Cantharidin reveals a significant difference at the concentrations of control and the maximum difference was in 5.5 µg ml^{-1} Comparison of the activity of glutathione peroxidase in the samples treated with cantharidin and endothall indicated that the maximum changes in the activity of this enzyme were observed in the samples treated with cantharidin.

In this research, the maximum extent of the increased expression of catalase and glutathione per-

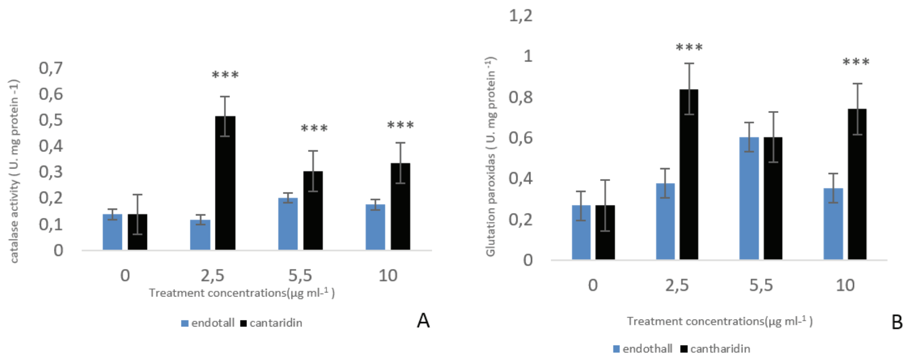


Figure 3: Changes in the activity of catalase enzyme in the shoot samples of *Chicorium intybus* L. plant treated with different concentrations of endothall and cantharidin (A), changes in the activity of glutathione peroxidase enzyme in the shoot samples of *Chicorium intybus* L. with different concentrations of endothall and cantharidin (B) ***Representing significant effects at 0.1 % probability level.

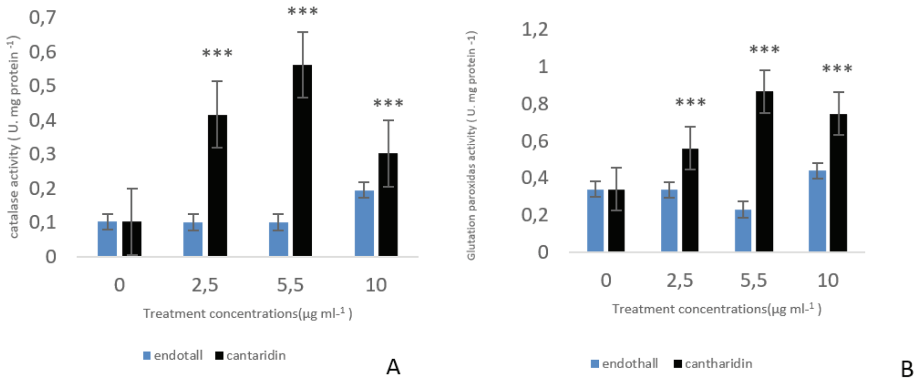


Figure 4: Changes in the activity of catalase enzyme in the root samples of *Chicorium intybus* L. plant treated with different concentrations of endothall and cantharidin (A), changes in the activity of glutathione peroxidase enzyme in the shoot samples of *Chicorium intybus* L. with different concentrations of endothall and cantharidin (B) ***Representing significant effects at 0.1 % probability level.

oxidase was observed in the treatment groups with the endothall concentrations of 5.5 and 2.5 µg ml^{-1}, as well as in the shoots of those with the cantharidin concentrations of 2.5 and 2.5 µg ml^{-1}. Furthermore, the maximum elevation of the gene expression of catalase in the roots of the samples treated with endothall and cantharidin was observed at the concentrations of 10 and 5.5 µg ml^{-1}, respectively. As for glutathione peroxidase, the maximum elevation was denoted at the concentration of 5.5 µg ml^{-1}.

In the mentioned treatment groups, the activity of catalase and glutathione peroxidase increased, suggests the onset of damage to the plant and activation of the antioxidant defense mechanism. In addition, treatment with cantharidin exerted more significant effects on the activity of catalase and glutathione peroxidase compared to endothall. In other words, the induction of damage to the plant initiated at lower concentrations, leading to a stronger response in the plant. However, the intensity and peak of the catalase and glutathione peroxidase activity declined at higher concentrations, which could be due to damage to the shoot or root cells since the elevated endothall and cantharidin concentrations may have partly eliminated the cellular response. Consistently, the results of the present study indicated that the level of antioxidant enzymes increased in the plants treated with cantharidin.

In this regard, Bajsa et al. (2012b) examined the effects of cantharidin on the biology of *Arabidopsis*. According to the obtained results, most of the protein detected

Table 2: Analysis of variance for the effect of endothall and cantaridin on shoot and root of *Chicorium intybus* L. under catalase treatment

	df	Shoot CAT	Root CAT
Model	15	0.140n.s	0.100n.s
Endotal 2.5	5	0.120n.s	0.100n.s
Endotal 5.5	5	0.200n.s	0.100n.s
Endotal 10	5	0.170n.s	0.190
Error	-	0.140	0.140
C.V.	-	7.49	4.39
Model	15	0.140n.s	0.100n.s
Cantaridin 2.5	5	0.515***	0.420***
Cantaridin 5.5	5	0.300***	0.560***
Cantaridin 10	5	0.330***	0.300***
Error	-	0.101	0.150
C.V.	-	8.14	7.28

Table 3: Analysis of variance for the effect of endothall and cantaridin on shoot and root of *Chicorium intybus* L. under glutation peroxidase treatment

	df	Shoot GPX	Root GPX
Model	45	0.270n.s	0.340n.s
Endotal 2.5	15	0.380n.s	0.330n.s
Endotal 5.5	15	0.600n.s	0.230n.s
Endotal 10	15	0.350n.s	0.440n.s
Error	-	0.071	0.040
C.V.	-	12.23	7.01
Model	45	0.270n.s	0.340n.s
Cantaridin 2.5	15	0.840***	0.560***
Cantaridin 5.5	15	0.600***	0.860***
Cantaridin 10	15	0.740***	0.740***
Error	-	0.061	0.040
C.V.	-	12.18	13.16

in the chloroplast diminished significantly, especially the proteins involved in photosynthesis. Conversely, the specific enzymes increased 1.5-3.3 times, especially glutathione transferase. Since the main role of glutathione transferase (antioxidant enzyme) is to induce defense and detoxification in plant tissues, this finding suggested that cantharidin may stimulate defense responses (Akram, 2012), and one of the defense modes is the election of antioxidant enzymes in the plant. In another study, the mechanism of action of endothall was investigated in plants. The biological tests for examining the physiological effects of endothall demonstrated that this herbicide exerted phytotoxic effects. Unlike other detrimental herbicides, endothall does not inhibit the polymerization of tubulin under experimental conditions. These effects have also been observed after treatment with the inhibitors of phosphatase protein, cantharidin, and okadaic acid (Tresch, 2011). Peksel (2013) found out that syntetic compounds as norcantharidin shows antioxidant activities in vitro condition.

According to the present study, high concentrations of endothall and cantharidin led to the inhibition of plant growth, which could be due to the inhibition of the cell cycle. At lower concentrations, the enhanced activity and expression of these antioxidant genes could be observed in plants, which may be due to the activation of the defense mechanism of the plant against harmful effects and oxidative stress (Tresch, 2011a). In another research, the findings indicated that the activation of the catalase and peroxidase enzymes by silver nitrate could cause these enzymes to cooperate in the removal of hydrogen peroxide. Additionally, this was reported to be the main cause of the initiation of the antioxidant defense, so that the inadequate response of an enzyme to silver nitrate could be compensated for through elevating the activity of another enzyme (Shabani & Saghirzadeh, 2015). Moreover, the present study showed that the antioxidant defense occurred at low concentrations and with higher intensity in the samples treated with cantharidin, which could be considered as an opportunity for the higher effectiveness of endothall. As reported by Tresch (2011b), the performance of endothall phosphatase anti-protein in plants is more effective compared to cantharidin. These effects may partly originate from the inhibition of the effects of cantharidin at low concentrations through the antioxidant defense system.

The findings of the present research indicated that endothall and cantharidin phytotoxins cause changes in the activity and expression of oxidative stress enzymes, such as catalase and glutathione peroxidase. These enzymes convert free radicals into water and are among the most active barriers against oxidative reactions in plant cells (Arora, 2002). As has been demonstrated in corn roots, endothall deviates cell division and the orientation of the microtubule structure, thereby disrupting the cell cycle in the prometaphase phase. In addition, endothall has been reported to exert other effects, such as the compression of cell nuclei, inhibiting the DNA synthesis phase, and inducing extra mutations in plant cells. These effects are closely correlated with oxidative stress (Tresch, 2011c). Li et al. (2014) use ROS signaling in mutations CAT2, it's an antioxidant enzyme model for study the contributed factors in ROS signaling (Mhamdi et al., 2010).As reported previously, herbicides cause damage to plants, which mostly occurs through the production of reactive oxygen species (ROS) like superoxide radical, OH radicals and H_2O_2 (Gill & Tutja, 2010). Accordingly, the activity of antioxidant enzymes, which is closely associated with ROS, is considered an index of various biological stress. High levels of antioxidants in a plant cells may provide a special stress resistance (Nohatto et al., 2016). Under such circumstances, the elevated activity of one or several antioxidants may occur, thereby stimulating the resistance (Akram, 2012a). The antioxidant enzyme´s roll in stress controls ROS gathering and limiting oxidative injuries (Nohatto et al., 2016a).

4 CONCLUSIONS

The present study aimed to compare the effects of endothall and cantharidin herbicides on the changes in the gene expression and activity of the catalase and glutathione peroxidase enzymes that disorder signaling pathways by blocking the protein phosphatase gene expression in shoot and root of this plant and decrease growth parameters. The findings of this present research show the catalase and glutathione peroxidase activity increased the samples treated cantharidin and endothall compared to the controls, suggesting the initiation of antioxidant activity in the plant.

5 REFERENCES

Abbas, Z. K., Saggu, S., Sakeran, M.I., Zidan, N., Rehman, H., & Ansari, A.A. (2015). Phytochemical, antioxidant and mineral composition of hydroalcoholic extract of chicory (*Cichorium intybus* L.) leaves. *Saudi Journal Biological Sciences*, *22*(3), 322-326. http://dx.doi.org/10.1016/j.sjbs.2014.11.015

Akram, N.A., Ashraf, M., & Al-Qurainy, F. (2012). Aminolevulinic acid-induced changes in some key physiological attributes and activities of antioxidant enzymes in sunflower (*Helianthus annuus* L.) plants under saline regimes. *Scientia Horticulturae*, *142*, 143-148. http://dx.doi.org/10.1016/j.scienta.2012.05.007

Arora, A., Sairam, R.K., & Srivastava, G.C. (2002). Oxidative stress and antioxidative system in plants. *Current Science Association*, *82*, 1227-1238. http://doi.org/10.2307/jstor.24107045

Bais, H.P., Ravishankar, G.A. (2001). *Cichorium intybus* L. cultivation, processing, utility, value addition and biotechnology, with an emphasis on current status and future prospects. *Journal of the Science of Food and Agriculture*, 467-484. https://doi.org/10.1002/jsfa.817

Bajsa, J., Pan, Z., & Duke, S.O. (2011). Transcriptional responses to cantharidin in Arabidopsis thaliana reveal the involvement of multiple signal transduction pathways. *Physiologia Plantarum*, 188–205. https://doi.org/10.1111/j.1399-3054.2011.01494.x

Bajsa, J., Pan, Z., Dayan, F.E., Owens, D.K. & Duke, S.O. (2012). Validation of serine/threonine protein phosphatase as the herbicide target site of endothall. *Pesticide Biochemistry and Physiology*, *102*(1), 38-44. https://doi.org/10.1016/j.pestbp.2011.10.007

Dayan, F.E., Watson, S.B. (2011). Plant cell membrane as a marker for light-dependent and light-independent herbicide mechanisms of action. *Pesticide Biochemistry and Physiology*, 182–190. https://doi.org10.1016j.pestbp.2011.09.004

Duke, S.O., Powles, S.B. (2008). Glyphosate: a once in a century herbicide. *Pest Management Science*, 319–325. http://doi.org/10.1002/ps.1518

Funk, V.A., Bayer, R.J., Keeley, R.J., Chan, R., Watson, L., Gemeinholzer, B., & Schilling, E. (2005). Everywhere but Antarctica: using a supertree to understand the diversity and distribution of the Compositae. *Biologiske Skrifter*, *55*, 343–374.

Gill, S.S., Tuteja, N. (2012). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, *48*(12), 909-930. *http://doi.org/10.1016/j.plaphy.2010.08.016*

Heimler, D., Isolani, L.Vignolini, P., & Romani, A. (2009). Polyphenol content and antiradical activity of *Cichorium intybus* L. from biodynamic and conventional farming. *Food Chemistry*, *114*(3), 765-770. https://doi.org/10.1016/j.foodchem.2008.10.010

Li, S.C., Mhamdi, A., Trotta, A., Kangasjärvi, S., & Noctor, G. (2014). The protein phosphatase subunit PP2A-B1γ is required to suppress day length-dependent pathogenesis responses triggered by intracellular oxidative stress. *New Phytologist*, *202*(1), 145-160. https://doi.org/10.1111/nph.12622

Mac, A., Nelson, C.J., & Sharp, R.E. (1992). Peroxidase activity in the leaf elongation zone of tall fescue. *Plant Physiology*, *99*(3), 294-878. https://dx.doi.org/10.1104.pp.99.3.872

Marschner, H., Cakmak, I. (1992). Mangnesium defninciency and high light intensity enhance activities of superoxide dismutase and glutathione reductase i bean leaves. *Plant physiology*, *98*(4), 1222-1227. https://dx.doi.org/10.1104/pp.98.4.1222

Mhamdi, A., Queval, G., Chaouch, S., Vanderauwera, S., van Breusegem, F., & Noctor, G. (2010). Catalase function in plants: A focus on *Arabidopsis* mutants as stress-mimic models. *Journal of Experimental Botany*, *61*(15), 4197-4220. https://doi.org/10.1093/jxb/erq282

Nohatto, M.A., Agostinetto, D., Langaro, A.C., de Oliveira, C., & Ruchel, Q. (2016). Antioxidant activity of rice plants sprayed with herbicides. *Pesquisa Agropecuária Tropical*, *46*(1), 28-34. https://doi.org/10.1590/1983-40632016v4638011

Peksel, A., Celik, C., Ocal, N., & Yanardag, R. (2013). Antioxidant and radical scavenging activities of some norcantharidin and bridged perhydroisoindole derivatives. *Journal of the Serbian Chemical Society, 78*(1), 15–25. https://doi.org/10.2298/JSC120123036P

Sanders, NA., Lee, MA. (2013). *Real time PCR: Advanced technologies and applications*. Caister Academic Press

Senderski, M.E. (2009). Praktyczny poradnik o ziołach i ziołolecznictwie herbs. *A practical guide to herbs and herbal medicine*. Warszawa, 102–103.

Tresch, S., Schmotz, J., & Grossmann, J. (2011). Probing mode of action in plant cell cycle by the herbicide

endothall, a protein phosphatase inhibitor. *Pesticide Biochemistry and Physiology*, *99*(1), 86-95. https://doi.org/10.1016/j.pestbp.2010.11.004

Ward, G.C., Joo, J., Ramien, M., & Ying, Y. (2017). Extensive chemical burns in a child from misuse of cantharidin: a case report. *Universiy of Ottawa Journal of Medicine*, *7*(1), 64-67. https://doi.org/10.18192/uojm.v7i1.1999

Young, D.K. (1984). Field studies of cantharidin orientation by *Neopyrochroa flabellata* (Coleoptera: Pyrochroidae). *Great Lakes Entomologist*, *17*(3), 1-4. https://scholar.valpo.edu/tgle/vol17/iss3/4

Navodila avtorjem

UVOD

Acta agriculturae Slovenica je četrtletna odprtodostopna znanstvena revija z recenzentskim sistemom, ki jo izdaja Biotehniška fakulteta Univerze v Ljubljani. Revija sprejema izvirne in še neobjavljene znanstvene članke v slovenskem ali angleškem jeziku, ki se vsebinsko nanašajo na širše področje rastlinske pridelave in živalske prireje in predelave. Zajema sledeče teme: agronomija, hortikultura, biotehnologija, fiziologija rastlin in živali, pedologija, ekologija in okoljske študije, agrarna ekonomika in politika, razvoj podeželja, sociologija podeželja, genetika, mikrobiologija, imunologija, etologija, mlekarstvo, živilska tehnologija, prehrana, bioinformatika, informacijske znanosti in ostala področja, povezana s kmetijstvom. Pregledne znanstvene članke sprejemamo v objavo samo po poprejšnjem dogovoru z uredniškim odborom. Objavljamo tudi izbrane razširjene znanstvene prispevke s posvetovanj, vendar morajo taki prispevki zajeti najmanj 30 % dodatnih izvirnih vsebin, ki še niso bile objavljene. O tovrstni predhodni objavi mora avtor obvestiti uredniški odbor. Če je prispevek del diplomske naloge, magistrskega ali doktorskega dela, navedemo to in tudi mentorja na dnu prve strani. Avtorji omenjenih del morajo biti tudi soavtorji članka, ki podaja izsledke dela. Navedbe morajo biti v slovenskem in angleškem jeziku, kadar so prispevki v slovenščini. V primeru člankov v slovenščini so dolžni avtorji priskrbeti angleški prevod naslova, izvlečka s ključnimi besedami in naslovov slik in tabel. Uredništvo revije zagotovi prevode izbranih bibliografskih elementov (naslova, izvlečka, opomb in ključnih besed) v primeru tujih avtorjev. Prispevke sprejemamo skozi celo leto samo preko spleta v OJS sistemu.

POSTOPEK ODDAJE PRISPEVKOV

Avtorji lektorirane prispevke oddajo v elektronski obliki na spletni strani OJS Acta agriculturae Slovenica.

Author guidelines

INTRODUCTION

Acta agriculturae Slovenica is an open access peer-reviewed scientific journal published quarterly by the Biotechnical Faculty of the University of Ljubljana, Slovenia. The journal accepts original scientific articles from the fields of plant production (agronomy, horticulture, plant biotechnology, plant-related food-and-nutrition research, agricultural economics, information-science, ecology, environmental studies, plant physiology & ecology, rural development & sociology, soil sciences, genetics, microbiology, food processing) and animal production(genetics, microbiology, immunology, nutrition, physiology, ecology, ethology, dairy science, economics, bioinformatics, animal production and food processing, technology and information science) in the Slovenian or English languages. Review articles are published upon agreement with the editor. Reports presented at conferences that were not published entirely in the conference reports can be published. Extended versions of selected proceedings-papers can also be considered for acceptance, provided they include at least 30 % new original content, but the editorial board must be notified beforehand. If the paper is part of a BSc, MSc or PhD thesis, this should be indicated together with the name of the mentor at the bottom of the front page and will appear as footnote. Authors of mentioned thesises should be also coauthors of manuscript. Slovenian-language translation of selected bibliographic elements, for example the title, abstract, and key words, will be provided by the editorial board. Manuscripts are accepted throughout the year. Only online submissions are accepted..

SUBMISSION PROCESS

Manuscripts should be submitted to the Acta agriculturae Slovenica OJS site. Complete manuscripts in-

Oddajo kompleten članek (naslov, avtorji z njihovimi naslovi, označen mora biti korespondenčni avtor in naveden njegov elektronski naslov, besedilo z vsemi poglavji in vključenimi ilustracijami (slike, tabele, enačbe). Pred oddajo prispevka se mora avtor na spletni strani najprej prijaviti oziroma registrirati (priporočamo, da je to korespondenčni avtor), če prvič vstopa v sistem (potrebno je klikniti na Registracija in izpolniti obrazec za registracijo). Bodite pozorni, da na dnu registracijskega obrazca ne pozabite odkljukati potrditvenega polja »Avtor«, sicer oddaja prispevka ne bo mogoča.

Postopek oddaje prispevka poteka v petih korakih. Priporočamo, da se avtor pred oddajo najprej seznani s postopkom in se na oddajo prispevka pripravi:

Korak 1: Začetek oddaje prispevka
- Izbrati je treba eno od sekcij,
- pri rubriki »Pogoji za oddajo prispevka« morate potrditi vsa potrditvena polja,
- dodatna pojasnila uredniku je mogoče vpisati v ustrezno polje.

Korak 2: Oddaja prispevka
- Naložite prispevek v formatu Microsoft Word (.doc ali .docx).

Korak 3: Vpis metapodatkov
- Podatki o avtorjih: ime, priimek, elektronski naslovi in ustanove vseh avtorjev v ustreznem vrstnem redu. Korespondenčni avtor mora biti posebej označen.
- Vpišite naslov in izvleček prispevka.
- Vpišite ključne besede (največ 8, ločeno s podpičjem) in označite jezik besedila.
- Vnesete lahko tudi podatke o financerjih.
- V ustrezno besedilno polje vnesite reference (med posameznimi referencami naj bo prazna vrstica).

Korak 4: Dodajanje morebitnih dodatnih datotek
- Za vsako dodatno naloženo datoteko je treba zagotoviti predvidene metapodatke.

Korak 5: Potrditev
- Potrebna je končna potrditev.

cluding title, authors and their affiliations, indicated corresponding author and his/her e-mail, abstract, text body of manuscript with recommended chapters and included illustration at proper sites within the text should be submitted regardless submitted metadata. The submitting author, preferably the corresponding author should be registered to the site. Click Register and fill in the registration form. Be sure to check in the Author check box on the form. We advise you to check in also the Reader check box.

Submission process consists of 5 steps. Before submission, authors should go through the checklist and prepare for submission:

Step 1: Starting the submission
- Choose one of the journal sections.
- Confirm all the requirements of the Submission Preparation Checklist.
- Additional plain text comments for the editor can be provided in the relevant text field.

Step 2: Upload submission
- Upload full manuscript in the form of the Microsoft Word document file format (.doc or .docx).

Step 3: Enter metadata
- First name, last name, contact e-mail and affiliation for all authors, in relevant order, must be provided. Corresponding author has to be selected.
- Title and abstract must be provided in plain text.
- Key words must be provided (max. 8, separated by semicolons) and enter the language of the text.
- Data about contributors and supporting agencies may be entered.
- References in plain text must be provided in the relevant text filed (between each reference should be a blank line).

Step 4: Upload supplementary files
- For each uploaded file the author is asked for additional metadata, which may be provided.

Step 5: Confirmation
- Final confirmation is required.

PODROBNEJŠA NAVODILA / DETAILED INSTRUCTIONS

http://ojs.aas.bf.uni-lj.si/index.php/AAS/about/submissions#authorGuidelines